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PLANT PHYSIOLOGY

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THE PROBLEM OF SELECTING UNIFORM SAMPLES OF LEAVES

HUBERT BRADFORD VICKERY, CHARLES S. LEAVENWORTH[1] AND CHESTER I. BLISS

Received January 22, 1949

In order to study the effect of cultural or other treatments upon the chemical composition of excised leaves, it is necessary to collect a number of separate samples from the plants. One or more of these is at once dried or otherwise prepared for analysis to serve as a control; the others are subjected to the treatment and are then prepared for analysis in exactly the same manner. The necessary chemical determinations are subsequently carried out and the differences in composition between the treated samples and the controls are computed; these differences are assumed to furnish a measure of the effect of the treatment. The validity of this assumption is, clearly, dependent upon the precision with which the samples duplicated each other in composition at the time of collection.

Experience in this laboratory has shown that samples which duplicate each other more or less closely can readily be obtained. Nevertheless, this general method of investigation is subject to errors that occasionally interfere with the interpretation of the results. The chief source of these errors appears to be the failure of occasional individual samples to agree sufficiently closely with the others in initial composition. Inasmuch as the analytical data cannot be obtained until after the experiment has been completed, there is no opportunity to repeat an erratic observation, for the original plant material has meanwhile changed in composition.

To avoid improper interpretations arising from such possible sampling errors, it has been customary to disregard chemical changes of less than about 10 per cent. of the magnitude measured. Changes greater than this are assumed to be the result of the treatment, but smaller changes may be open to the suspicion of being affected by the sampling error.

The error in an analytical determination obtained upon plant material has two main components. One of these arises from variations in the analytical processes in the laboratory. This error can be estimated from the differences between the results of analytical determinations obtained in duplicate. The other component, arising from actual variations of the plant material itself, is open to study if different techniques of sampling are employed. Analysis of the data, after segregation of the analytical

[1] Died Nov. 20, 1948.

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error, should then lead to judgments on the relative desirability of one or other of these sampling techniques.

In an effort to obtain objective evidence upon the magnitude of the sampling error in the selection of presumably similar samples of leaves, three methods of collection have accordingly been studied, *Bryophyllum calycinum* being used as the species investigated.

Methods of sampling

STATISTICAL METHOD.—The objective of the statistical method of sampling is to equalize the effects of the differences between the plants and the differences between leaf positions on the individual plants. The samples are so taken that each plant and each leaf position is represented to exactly the same extent in each sample. Because of the necessity of providing an exact balance of the several factors, there are restrictions on the relative numbers of samples, of plants, and of leaf positions that can be used.

The number of categories of leaf position that it is proposed to study is first established and a number of plants that is an integral multiple or submultiple of this number is selected. A number of samples that is also an integral multiple or submultiple of this number is then fixed upon. The selected plants are examined to make sure that each of the leaves or leaflets to be collected is normally developed and free from injury. The plants are assigned numbers at random, and an arbitrary plan of numbering the leaves or leaflets, *i.e.* the categories of leaf position, is set up. For example, with the Bryophyllum plants used in this study, each of the five leaflets on a pair of opposite leaves high on the plant was assigned the numbers 1 to 10 according to an easily remembered scheme. This system of numbering was then applied to the leaflets of the pair of opposite leaves in the same relative position on the stalks of each of the five plants used.

At the time of collection, leaflet 1 from plant 1 is placed in sample 1, leaflet 2 from plant 1 in sample 2, leaflet 3 from plant 1 in sample 3, and so on. If there are 5 samples, leaflet 6 will go into sample 1; if 10, the leaflets will go once around the set of samples. On plant 2, leaflet 1 is placed in sample 2, leaflet 2 in sample 3, and so on. On plant 3, leaflet 1 is placed in sample 3, etc. This progressive process is continued throughout the group of plants. To obtain larger samples, a second pair of leaves adjacent in position to the first pair is then treated in the same way and a third or fourth pair may also be included if desired.

The design employed is, in mathematical terms, a systematized Latin square and is selected for the present purpose to facilitate the picking of the samples. Alternatively, an ordinary Latin square in which the numbering of the leaflets is entirely randomized may be used but is more difficult to employ. A diagram of the system as it applies to the leaflets from one leaf on each of five plants is as follows.

Sample numbers to which designated leaflets are alloted according to a systematic Latin square designed to give 5 samples

Leaflet position	Plant number				
	1	2	3	4	5
	Sample number				
1	1	2	3	4	5
2	2	3	4	5	1
3	3	4	5	1	2
4	4	5	1	2	3
5	5	1	2	3	4

Leaflets 6 to 10 from the first pair of opposite leaves are collected according to a similar plan. In collecting the leaflets from the second pair of opposite leaves, the sampling plan may be identical, but, in the present case, was modified by setting up the Latin square beginning with sample number 2 in the upper left-hand corner and the third pair beginning with sample number 3 in the upper left-hand corner. Any other number from 1 to 5 may equally well be chosen. This is equivalent to extending the Latin square shown into a third dimension.

When the collection is finished, there is an equal number of leaflets in each sample, and each sample represents equally all of the plants, all of the leaflet positions on the leaves, and all of the leaf positions chosen. Accordingly, if the variability in the composition of leaves is assumed to be made up of a component that varies from plant to plant, one that varies with leaf position, one that varies with leaflet position, and an additional random component, each of these components is distributed evenly among the samples.

It is obvious that the 5-leaflet upper compound leaves of mature *Bryophyllum calycinum* plants restrict the number of samples that can be obtained by this method to 5 or a multiple of 5. If younger plants are used in which the upper compound leaves bear only 3 leaflets, a number of samples that is a multiple of 3 may be collected from this species. In this case, the number of plants used must also be a multiple of 3. In any case, although collection of the full mathematically determined number of samples is necessary, some of them may be omitted from the experiment, or the extra samples may be used for the duplication of controls or treatments.

OPPOSITE LEAF METHOD.—This method has long been used in physiological experiments (*e.g.* CHIBNALL (**2**)), on the assumption that the composition or behavior of opposite leaflets would, for morphological reasons, be so similar that one can properly be used as a control in studying the effect of treatment upon the other. In selecting the samples, there is no restriction upon the number of plants or the number of pairs of opposite leaflets employed. However, the number of samples must be evenly divisible by the number of categories of leaflets established. Differences between plants are balanced in that each sample contains an equal number

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of leaflets from each plant. However, each sample consists of leaflets from the same position on the plant.

In selecting the samples, a suitable number of pairs of compound leaves in the same positions on a sufficient number of plants is selected and examined for freedom from abnormality and for similarity in appearance and development. In the present experiment with *B. calycinum*, the strict application of the method was slightly modified in that five categories of leaflets were set up: the first comprised the two leaflets on one side of one of a pair of similarly placed opposite leaves on each plant; the second, the leaflets opposite to these; the third, the two leaflets on one side of the opposite leaf; and the fourth, the two leaflets opposite these. A fifth category was made up of the terminal leaflets. In making the collection, all of the leaflets of the first category from each of the plants are placed in one sample, all of the second category in the next and so on, and a second and, if desired, third pair of leaves from all of the plants are treated in the same manner. At the end, each sample contains the same number of leaflets, and the plants are equally represented in each sample. Samples 1 and 2 represent mutually opposite leaflets as do samples 3 and 4. The fifth sample taken in the present case was, however, unique with respect to leaf position and would not be employed in an experiment designed to conform strictly to the opposite leaf method. However, its composition proved to be indistinguishable from the others and it was included to facilitate the mathematical analysis.

LEAF SIZE METHOD.—This method may be used with any species the leaves of which are of suitable size. It provides for randomization with respect to plant of origin and position on the plant. The plants are selected and a decision is made on the numbers and position of the leaves to be collected. There is no restriction on the number of plants nor upon the number of samples. However, randomization is best provided for in large samples. The leaves or leaflets are collected into a single receptacle, care being taken to collect a few more than will be required.

The correct total number of leaves for the set of samples is then counted out, discarding and replacing abnormal or damaged leaves, and the leaves are sorted according to size and degree of development, the number of grades set up depending on the variation in size. The leaves in each grade are then shuffled repeatedly so as to randomize them as much as is conveniently possible, and the number of leaves in each grade is noted. Where this number is not evenly divisible by the number of samples to be taken, adjustment is made by moving leaves of suitable size from one grade to another. After further shuffling, the leaves in each grade are distributed equally but at random to the samples.

The leaf size method has been used for many years in this laboratory. However, the sampling error problem that has occasionally arisen suggests that the degree of randomization that is attained in practice is not always adequate.

Experimental

Five samples of leaflets of *B. calycinum* were taken by each of the three methods described. There were 30 leaflets (five plants, three pairs of opposite leaves) in each sample taken by the statistical method, 36 (six plants, three pairs of opposite leaves) in each sample taken by the opposite leaf method, and 30 (150 leaflets from upper leaves of about 10 plants) in each sample taken by the leaf size method. The samples were collected by the statistical and opposite leaf methods in the early afternoon of a sunny day so that the organic acids would be at a low point and starch at a maximum (**7**). Those taken by the leaf size method were collected three weeks later also on the afternoon of a sunny day but after a prolonged period of cloudy weather.

Each set of samples was immediately dried at 80° C., equilibrated at

TABLE I

ANALYTICAL DATA GIVING DUPLICATE DETERMINATIONS IN GRAMS PER KILO OF INITIAL FRESH WEIGHT FOR EACH ALIQUOT OF POWDERED LEAF. SAMPLES IN SERIES ST. COLLECTED BY THE STATISTICAL METHOD, IN SERIES O.L. BY THE OPPOSITE LEAF METHOD, AND IN SERIES L.S. BY THE LEAF SIZE METHOD

Series	Sample No.	Total solids		Ash		Total nitrogen		Protein nitrogen		Starch	
St.	1	110.2	110.0	10.07	10.18	2.79	2.84	2.32	2.32	25.35	25.35
	2	105.9	105.8	9.85	9.87	2.78	2.78	2.23	2.24	24.35	24.35
	3	106.7	106.7	10.41	10.34	2.87	2.83	2.26	2.26	24.50	24.84
	4	105.9	106.2	9.96	10.28	2.73	2.75	2.23	2.23	25.47	25.02
	5	107.8	107.5	10.09	10.36	2.79	2.79	2.27	2.27	25.57	25.57
O.L.	1	110.2	110.1	10.77	10.40	3.05	3.05	2.43	2.42	24.05	24.05
	2	104.5	104.2	11.23	10.94	2.87	2.87	2.31	2.30	22.43	22.43
	3	106.2	105.9	11.20	11.27	2.91	2.91	2.31	2.30	23.64	23.25
	4	102.7	102.8	10.23	10.19	2.76	2.79	2.28	2.27	22.66	23.06
	5	108.6	108.6	10.24	10.10	2.81	2.81	2.31	2.30	23.99	23.99
L.S.	1	118.0	118.3	11.98	10.84	2.48	2.48	2.03	2.04	32.08	32.08
	2	121.6	121.7	11.69	11.79	2.59	2.63	2.06	2.05	33.50	33.50
	3	128.9	128.9	12.43	12.21	2.47	2.51	1.98	1.98	42.33	42.33
	4	119.4	119.4	11.30	11.23	2.37	2.41	1.92	1.92	35.83	35.83
	5	125.1	125.4	11.76	11.92	2.48	2.48	2.04	2.04	38.14	37.61

25° C. and 50 per cent. relative humidity in a constant temperature-constant humidity room until constant weight was attained and was then ground to powder in a Wiley mill for analysis.

The analytical determinations shown in table I were made, the data being expressed in grams per kilogram of fresh weight of the sample at the time of collection. The total solids were computed from the loss of weight of duplicate 500 mg. aliquots of the equilibrated powdered tissue when heated at 110° C. for exactly four hours. The same samples were used for the determination of the ash as described by VICKERY, PUCHER, WAKEMAN and LEAVENWORTH (**9**). Total nitrogen was determined on 100 mg. samples by the Kjeldahl method modified to deal with nitrate as described by VICKERY, PUCHER, WAKEMAN and LEAVENWORTH (**9**), except that a

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loose plug of nonabsorbent cotton was placed in the neck of the Kjeldahl flask during the distillation as an additional precaution against entrainment of minute drops of alkali, and that the flask used to collect the distillate was cooled in a bath of cold water during the distillation.

Protein nitrogen was determined in duplicate 200 mg. samples by a recently developed procedure according to which the samples, securely wrapped in small squares of closely woven cotton fabric, are extracted with 70 per cent. alcohol in a continuous extraction apparatus for 16 hours (**6**). They are subsequently transferred to a centrifuge tube and extracted with hot water for 10 minutes and centrifuged. The extract is decanted through a filter paper and the residue is then washed quantitatively into a Kjeldahl flask and total nitrogen is determined. It is assumed that the nitrogen that remains insoluble under these conditions represents protein nitrogen.

Starch was determined by the method of PUCHER, LEAVENWORTH and VICKERY (**8**).

Statistical analysis of the data

A preliminary examination of the data for each constituent was made in terms of the coefficients of variation of the means of the duplicate determinations. In each case, the variation within the group of samples collected by the statistical method was less than within either of the other two groups, suggesting the superiority of this method, but no choice could be made between the other two methods. However, the results showed sufficient promise to warrant a more extended analysis.

The study concerned the relative variability of each component for each method of collection, so that the determinations in table I were transformed to logarithms for analysis. All of the computations reported here have been based upon these log values. Their means have been transformed back into the original units to obtain the "geometric mean content" of each constituent shown in table II.

The first step was to estimate the analytical error for each constituent from the differences in the five paired values in each of the three series of samples. The standard deviation in units of a single observation was computed initially for each series and the series compared. As would be expected, they did not differ significantly from one another. The variances for each constituent were then pooled, the square roots of these composite values representing standard deviations, each with 15 degrees of freedom. The percentage analytical errors in column 6 table II are the antilogarithms of each such standard deviation, diminished by 1 and multiplied by 100.

The differences in the paired determinations for percentage of ash were markedly more variable than those for any other constituent. Even with the most discrepant difference omitted, the percentage standard deviation from the remaining 14 values dropped only from 2.24 to 1.30 per cent. and the latter value was still nearly double that for the next most

variable constituent.[2] The other analytical errors varied considerably more from one another than would be expected by chance if, in fact, all were equally precise. The analytical error for the protein nitrogen was less than that for total nitrogen, but the differences in the analytical error could be attributed only in part to the larger aliquots used for protein nitrogen.

There is a possibility that the analytical error of these determinations is underestimated because of the custom of the analyst of weighing out the samples for duplicate determinations at the same time and carrying out the analyses in pairs. This practice carries with it the possibility of a subconscious bias when, for example, the titration of the second member of an identical pair is made. In point of fact, many of the duplicate determina-

TABLE II

COMPOSITION OF SAMPLES OF LEAVES OF *Bryophyllum calycinum* IN GRAMS PER KILO OF INITIAL FRESH WEIGHT. SAMPLES IN SERIES ST. COLLECTED BY STATISTICAL METHOD, IN SERIES O.L. BY OPPOSITE LEAF METHOD, AND IN SERIES L.S. BY LEAF SIZE METHOD. ERRORS REPRESENT STANDARD DEVIATIONS

CONSTITUENT	SAMPLE SIZE	GEOMETRIC MEAN CONTENT			ANALYTICAL ERROR	NET SAMPLING ERROR		
		ST.	O.L.	L.S.		ST.	O.L.	L.S.
	mg.	*gm. per kilo*	*gm. per kilo*	*gm. per kilo*	%	%	%	%
Total solids	500	107.26	106.34	122.60	0.14	1.61	2.89	3.64
Ash	500	10.14	10.65	11.71	2.24*	1.01	4.40	3.20
Total nitrogen	100	2.795	2.881	2.489	0.70	1.39	3.71	3.14
Protein nitrogen	200	2.263	2.322	2.005	0.23	1.59	2.51	2.85
Starch	100	25.03	23.35	36.15	0.66	2.00	3.04	11.48†

* Omitting the most discrepant pair in 15 reduces this value to 1.30 per cent.
† In subsequent statistical analysis, this value was replaced by 3.71 per cent. See text for explanation.

tions were exactly equal to the limits of observation. The data would have been improved for the present purpose if single determinations had been made throughout a given set of samples and then repeated. Furthermore, the statistical analysis would have been facilitated if an effort had been made to estimate an additional significant figure in the measurements.

Although separate aliquots were used for each constituent (except ash), some correlation might be expected among the five measured constituents of each sample. An analysis of variance was computed for each method of sampling, separating the variation among the totals of the five constituents in each sample (with 4 degrees of freedom) from the interaction of samples with constituents (with 16 degrees of freedom). In every case

[2] The difficulty of obtaining high precision in the determination of ash in plant tissues has long been recognized and much study has been given to the problem in this laboratory. It seems possible that some of the trouble arises from the instability of calcium carbonate at the temperature ($590 \pm 10°$ C.) of the muffle furnace.

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the sample totals were the more variable. The variance ratios (F) for series St., O.L., and L.S. were 4.40, 3.56, and 2.52, respectively, the first two being statistically significant. These F ratios may also be expressed as intra-class correlations (5) with an average value of $r = 0.327$ on a scale of 0 to 1. Although the relative amounts of the constituents in each sample were correlated significantly, the correlation was small and has been disregarded in comparing the sampling variances of the different methods.

As has been mentioned, the variation in a constituent from sample to sample involves two components. The first is the analytical error which has already been measured; the second is the variation among samples within a series. In an analysis of variance, the mean square between duplicates within samples is an estimate of the first component, *i.e.*, the analytical error, but that among samples includes both components. In the present study, the net variance among samples could be isolated and

TABLE III

ANALYSIS OF VARIANCE OF NET SAMPLING ERRORS, COMPUTED IN UNITS OF THE LOGARITHM OF THE NET VARIANCE COMPONENT AMONG SAMPLES, DETERMINED FROM FIVE LOG-WEIGHTS OF EACH CONSTITUENT. DISCREPANT STARCH VARIANCE (SERIES L.S.) REPLACED BY MISSING PLOT TECHNIQUE

TERM	DEGREES OF FREEDOM	VARIANCE RATIO, F
Among constituents	4	0.36
Statistical samples vs. others	1	35.46
Opposite leaf vs. leaf size samples	1	0.01
Effect of discrepant starch variance on error	1	11.09
Residual error	7	1.00

was equal to one-half of the difference between two mean squares, that among samples and that between duplicates within samples (3). The net variances among samples were changed to square roots and the square roots to antilogarithms to obtain the percentage errors shown in the last three columns of table II, each with 4 degrees of freedom. It is evident by inspection that the statistical method of leaf sampling gave a smaller net sampling error than either the opposite leaf or the leaf size method. The results are generally consistent except that the starch determinations from the leaf size samples had an abnormally large sampling error. This set of samples was not taken concurrently with the other two sets, and intervening weather conditions may have been a factor in the greater variability in the starch content of these leaves.

To determine the significance of the variations in the net sampling error, an analysis of variance was computed from the logarithms of the net variances among samples, which, in turn, were computed from the log-weights of constituent. As is shown by BARTLETT and KENDALL (1), the variance in these terms conforms better to the hypotheses underlying the

analysis of variance. Two analyses were computed, one with the observed values and one in which the starch estimate for series L.S. was replaced by the missing plot technique (**4**). This substitution reduced the estimate of error so markedly that it has been used in table III, although substantially the same results were obtained with either the original or the substituted value.

From the variance ratios in table III, it is evident that the leaf samples varied about equally in each constituent. The samples collected by the statistical method were clearly more uniform ($P < .001$) than the samples collected by either of the other two techniques. The opposite leaf and leaf size samples, on the other hand, were equally variable. For measurements of leaf constituents of the same precision, from four to five times as many leaf samples would be needed if collected by either of these two methods instead of by the statistical technique.[3] The relatively large effect of the discrepant starch variance in the leaf size sample is evident from the variance ratio in the fourth row of table III.

Summary

The uniformity in composition of samples of leaves of *Bryophyllum calycinum* has been tested by statistical analysis of the data obtained in duplicate for solids, ash, nitrogen, protein, and starch. Three sets of five samples each were collected, one by a method, referred to as the statistical method, which balances equally among the samples the effects of differences among plants, leaf positions, and leaflet positions. A second set was collected by a modification of the opposite leaf method and a third by a method of randomization according to leaf size.

A comparison of the results of the duplicate determinations showed that the several analytical procedures were of unequal reliability, that for ash being the least precise. After segregation of the analytical error, the net variation among samples was determined for each method of collection and for each constituent. The five constituents showed no differences in variability and the statistical method gave the most uniform samples. To obtain by either of the other two methods the same precision as was secured by the statistical method, approximately four to five times as many samples would be needed.

BIOCHEMISTRY LABORATORY
CONNECTICUT AGRICULTURAL EXPERIMENT STATION,
NEW HAVEN, CONNECTICUT

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[3] This estimate is based upon the geometric means of the corresponding net sampling variances of the log-weights, which were 0.000041 for the statistical method and 0.000195 for the other two techniques. The number of samples required for equal precision is inversely proportional to these estimates.

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GROWTH AND COMPOSITION OF BILOXI SOYBEAN GROWN IN A CONTROLLED ENVIRONMENT WITH RADIATION FROM DIFFERENT CARBON-ARC SOURCES

M. W. Parker and H. A. Borthwick

(WITH TWO FIGURES)

Received March 21, 1949

Introduction

Controlled environment rooms in which reasonably large populations of plants can be grown under reproducible environmental conditions are advantageous for many kinds of plant research and are a vital necessity for certain types. One of the chief technical problems connected with the construction of such equipment arises from the fact that no source of artificial visible radiation that is completely satisfactory for the growing of plants has yet been developed. Many kinds of lamps have been tested and although all have objectionable features two types, the carbon-arc and the fluorescent, have been found reasonably satisfactory. One type or the other is the principal source of radiation for most of the controlled environment rooms now in operation.

At the Plant Industry Station, Beltsville, Maryland, four environmental control rooms have been in use since 1937. The principal radiation source in these rooms is an alternating current carbon-arc lamp. The lamps burn "Sunshine" carbons that are cored with materials which greatly increase the energy in the visible spectrum and particularly in the blue-violet end. Radiation from such carbons does not duplicate solar radiation, but reasonably satisfactory growth of soybeans can be obtained with this as the only source of radiant energy. Soybeans grown with radiation emitted by "Sunshine" carbons have repeatedly been found to have a somewhat lower carbohydrate content than soybeans grown with solar radiation. A possible cause of this was the fact that visible radiation from "Sunshine" carbons contains a smaller proportion of its energy in the red end of the spectrum than does solar radiation. This deficiency was partially corrected by supplementing the radiation from the arc lamp with that from incandescent-filament lamps, the visible radiation from which was largely in the red end of the spectrum. With this combined source the sturdiness of the plants was greatly improved and the carbohydrate content was increased. This combined source was adopted for regular use in the rooms described above and it has been possible with it to grow soybeans superior in quality and yield to those that can be grown during much of the year in the greenhouse. Since the irradiance at the surface of the plant is not more than 2000 foot-candles, of which only 160 foot-candles or considerably less than 10 per cent. is from the incandescent-filament lamps, the general improvement in quality and composition

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of the plants was assumed to have resulted from a peculiarity of the spectral distribution.

The installation of incandescent lamps in controlled environment rooms may not be desirable in some cases. Therefore, if the spectral distribution of "Sunshine" carbons could be altered to include sufficient red and still retain its other qualities a single source of light adequate for plant growth would be available. This has been attempted by varying the composition of the core of the carbon in such a way that it will emit more red radiation. The results have been evaluated by comparing the yield and chemical composition of soybeans grown with radiation from "Sunshine" carbons with those of soybeans grown with radiation emitted by various kinds of experimental carbons.[1] Additional comparisons have been made between plants grown with radiation from arc lamps and those that were grown with radiation from arc lamps in combination with incandescent-filament lamps.

Description of controlled environment rooms and culture of plants

The controlled environment rooms (fig. 1) were constructed as standard refrigeration rooms and were 18 feet long, 9 feet wide, and 10 feet high. An alternating current arc lamp was located in the center of each room at a height such that the flame of the arc was 6 feet from the floor. These arc lamps were operated at 75 amperes and 55 volts. Each lamp was equipped with a spun aluminum parabolic reflector which gave a relatively uniform irradiance over a circular area of 9-foot diameter beneath each lamp, except for an area 3 feet in diameter directly beneath the lamp, which was shaded and unsatisfactory for plant growth. The arc was enclosed by frames fitted with window glass which filtered out the ultraviolet radiation below 3100 A. Air was continuously drawn through the lamp housing to remove toxic fumes. In addition to the arc lamp each room had eight 200-watt incandescent-filament lamps arranged in a circle over the plant benches. The benches, which were semicircular in shape, had an outside diameter of 9 feet and were 3 feet wide. Two-gallon culture crocks were placed in three concentric circles on the benches. The height of the circles increased slightly from the inner to the outer in order to maintain the same intensity at the surface of each pot. Each room had a capacity of 58 crocks. Four plants were grown in each crock, which made a total of 232 plants per room.

Sand culture was employed and the crocks were flushed daily with a four-salt nutrient solution. Molar concentrations of the salts used in the nutrient solutions were .0058M $MgSO_4 \cdot 7H_2O$; 003M $CaCl_2 \cdot 2H_2O$; .0015M KH_2PO_4; and .0037M $NaNO_3$. In addition to these major elements, modified HOAGLAND (**5**) minor element solutions A and B were added at the rate of 50 ml. of solution A and 5 ml. of solution B for 50 L. of nutrient.

Two rooms were devoted to each experiment, one room with radiation

[1] All experimental carbons were furnished by the National Carbon Company.

from either "Sunshine" or special carbons alone and the other with combined radiation from incandescent-filament lamps and one of the carbon-arc sources. The next succeeding experiment reversed the arrangement. Even with a closely controlled environment, differences between experi-

FIG. 1. Controlled environment rooms showing Biloxi soybean plants grown in sand culture with 16-hour photoperiods. Photographed four weeks after planting. The principal source of radiant energy was an alternating current arc lamp burning "Sunshine" carbons. Upper figure shows soybeans grown with arc radiation supplemented with incandescent radiation. Lower figure shows soybeans grown in arc radiation without the supplemental incandescent radiation.

ments occurred, due to such factors as age of seed and minor differences in rooms. Thus in the first experiment a comparison was made between the growth and composition of Biloxi soybean grown with arc lamp using

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"Sunshine" carbons supplemented with incandescent-filament lamps and plants that were grown with arc lamps using experimental lower carbons, No. 025, which emitted more red (fig. 2) than "Sunshine" carbon. In the second experiment the incandescent-filament lamps were used with the

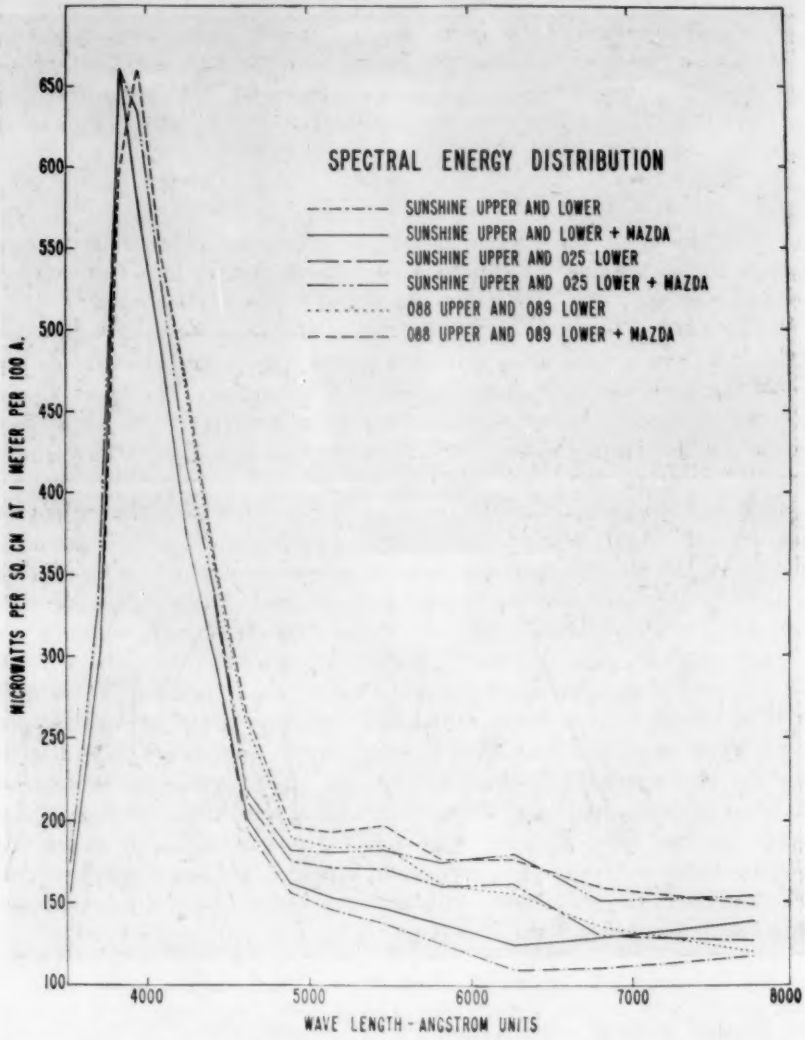

FIG. 2. -Spectral energy distribution of radiation from a carbon-arc lamp burning various carbons, with and without supplemental incandescent radiation. Data for graphs furnished by National Carbon Company.

025 carbons instead of with the "Sunshine." This comparison was made to determine whether or not the plants grown with 025 carbons would benefit from a further addition of red energy supplied by the supplemen-

tal incandescent lamps. In the third and fourth experiments both upper and lower experimental carbons, nos. 088 and 089, were used in one of the arc lamps. These carbons supplied more energy in the red region than the single pair of 025 experimental carbons, and their effectiveness was compared alone and in combination with incandescent-filament lamps. In the third experiment the incandescent-filament lamps were used with "Sunshine" carbons, and in the fourth with the 088 and 089 carbons. In all of the experiments the plants were grown for four weeks from time of seeding. The temperature was maintained at $70° \pm 1.5°$ F., and the photoperiod at 16 hours.

Sampling and chemical methods

The length of the first internode and the length from the primary leaves to the terminal of each stem were measured on the afternoon preceding harvest. The plants were harvested 28 days after seeding. Harvesting occurred at 8:00 A.M. following the usual 8-hour dark period. The stem was cut at the cotyledonary node, the leaves and the petioles were removed, and all primary leaves were discarded. The fresh weights of the stems and leaves were determined separately. The leaves were ground with a meat chopper. The stems were cut into quarter-inch lengths with a slicing machine and were then shredded in a rotary food chopper. Samples of the ground tissue were immediately taken for moisture, sugars, starch, and soluble non-protein nitrogen determinations. The method of sampling and the chemical methods employed were those reported previously (**8, 9**) except for the following deviations. Total sugars were determined by the Invertase Method (**2**) and the starch samples were preserved in 80 per cent. alcohol. The starch samples were extracted with 80 per cent. alcohol by decanting and washing on a Buchner funnel until free of sugars and were then ground to 100 mesh. The ground samples were again extracted with 80 per cent. alcohol and ether. The method reported for starch (**8, 9**) was then followed. The ammonia, nitrate, soluble non-protein, and total nitrogen fractions were determined as previously reported (**8**). Soluble organic nitrogen was calculated as the difference between the soluble non-protein nitrogen and the inorganic nitrogen, and the difference between total nitrogen and soluble non-protein nitrogen was designated protein nitrogen.

Results

When "Sunshine" carbon-arc irradiation was supplemented with incandescent-filament irradiation the plants were sufficiently sturdy and well developed that they did not require staking for the 4-week period (fig. 1). When the supplemental incandescent-filament lamps were not used the stems of the plants were less rigid and some of them fell during the fourth week when growth was most rapid.

The plants grown with radiation from the 025 experimental lower and

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"Sunshine" upper carbon did not have as much dry weight as those grown with radiation from the combination of "Sunshine" carbons and incandescent, even though there was only a slight difference in the length of the stems (table I).

In experiment 2 (table I) when the 025-"Sunshine" carbon combination was supplemented with incandescent-filament radiation the plants had more dry weight and longer stems than those grown with only "Sunshine" carbon-arc radiation. These plants were also heavier and taller than those grown with incandescent-supplemented "Sunshine" carbon-arc irradiation in experiment 1.

The general appearance of the plants (Expt. 3) that received radiation from the 088 and 089 carbons was the same as that of the plants which received energy from "Sunshine" carbons supplemented with the incandescent-filament lamp. However, the latter accumulated more dry weight in both leaves and stems and were slightly taller than the former.

When the radiation from the 088 and 089 carbons was supplemented with incandescent-filament lamps in experiment 4, the plants grew luxuriously. Dry weight accumulation in the leaves and stems was greater than in the plants grown with radiation from the "Sunshine" carbon-arc lamp and equaled or surpassed that of the plants grown with the combination of incandescent and "Sunshine" carbon-arc. The average stem length of the plants grown with the incandescent-supplemented, 088 and 089 carbon-arc was slightly greater than that of plants grown with the "Sunshine" carbon-arc.

The uniformity in the length of stems and in component parts is to be noted since these separate experiments were run at intervals extending over a two-year period. The maximum difference in the average length of the stem from the primary leaves to the terminal is 6.3 cm. These results indicate the high order of reproducibility of growth of soybeans in the controlled environment rooms employed.

Ammonia and nitrate nitrogen were determined for both leaf and stem samples in all of the experiments but these values are not shown in the tables. The percentage of ammonia was within the range normally found in soybeans and there were no significant trends or fluctuations. Nitrate nitrogen was adequate in all of the experiments and the percentage in the leaves and stems was normal for soybeans. There was however a slight tendency for the total inorganic nitrogen fractions to be greater in the plants that were grown with arc irradiation only. The percentage of soluble organic nitrogen and protein nitrogen in the leaves and stems varied within the limits usually found in four-week-old soybean plants grown in the controlled environment rooms (table II). On a milligram per plant basis the soluble organic nitrogen and the protein nitrogen in the leaves and stems were greater when a combination of arc and incandescent-filament lamps was used than when only the arc lamp was used. This can be accounted for through the greater dry weight of the plants grown under the former conditions.

TABLE I

AVERAGE WEIGHT AND HEIGHT OF BILOXI SOYBEANS GROWN FOR FOUR WEEKS ON 16-HOUR PHOTOPERIODS AT 70° F. WITH DIFFERENT SOURCES OF RADIATION. PLANTS HARVESTED AFTER AN 8-HOUR DARK PERIOD

Expt. No.	Sources of radiation		Percentage dry weight		Average fresh weight per plant		Average dry weight per 100 plants		Average length of stems		
	Carbons burned in arc lamp	Tungsten-filament lamps	Leaves	Stems	Leaves	Stems	Leaves	Stems	1st internode	Primary leaves to terminal	Total length
			%	%	*gms.*	*gms.*	*gms.*	*gms.*	*cm.*	*cm.*	*cm.*
1	"Sunshine"	8,200-watt	13.83	11.56	9.97	5.37	137.88	62.07	9.03 ± .09	19.60 ± .26	28.67 ± .30
	"Sunshine" upper No. 025 Lower	None	13.26	10.21	8.70	4.53	115.36	46.25	8.23 ± .10	19.38 ± .21	27.62 ± .26
2	"Sunshine"	None	12.76	9.87	7.57	4.19	96.59	41.35	8.64 ± .09	18.13 ± .14	26.77 ± .23
	"Sunshine" upper No. 025 Lower	8,200-watt	13.42	11.40	11.48	6.07	154.06	69.19	8.89 ± .10	23.60 ± .29	32.49 ± .33
3	"Sunshine"	8,200-watt	12.88	10.38	12.81	7.33	164.99	76.08	8.61 ± .07	24.45 ± .24	33.06 ± .29
	Nos. 088 and 089	None	12.20	10.07	10.20	5.45	124.44	54.88	8.13 ± .08	20.89 ± .20	29.02 ± .25
4	Nos. 088 and 089	8,200-watt	13.60	11.29	12.31	6.92	167.41	78.12	8.97 ± .07	22.61 ± .25	31.58 ± .29
	"Sunshine"	None	12.40	10.32	8.87	5.08	109.98	52.42	9.03 ± .11	20.08 ± .19	29.11 ± .25

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TABLE II

EFFECT OF VARIOUS RADIATION SOURCES ON THE NITROGEN CONTENT OF BILOXI SOYBEANS GROWN ON 16-HOUR PHOTOPERIODS AT 70° F. FOR FOUR WEEKS. PLANTS HARVESTED AFTER AN 8-HOUR DARK PERIOD

Expt. no.	Source of radiation		Percentage dry weight				Milligrams per plant			
	Carbons burned in arc lamp	Tungsten-filament lamps	Soluble organic nitrogen		Protein nitrogen		Soluble organic nitrogen		Protein nitrogen	
			Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems
			%	%	%	%	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	"Sunshine"	8,200-watt	1.137	1.759	4.288	1.453	15.67	10.91	59.13	9.02
	"Sunshine" upper No. 025 Lower	None	0.951	1.638	4.457	1.470	10.98	7.57	51.41	6.80
2	"Sunshine"	None	1.261	2.064	4.515	1.531	12.18	8.53	43.61	6.33
	"Sunshine" upper No. 025 Lower	8,200-watt	0.924	1.498	4.144	1.351	14.24	10.37	68.84	9.35
3	"Sunshine"	8,200-watt	1.291	2.187	4.326	1.340	21.30	16.64	71.38	10.19
	Nos. 088 and 089	None	1.123	1.905	4.474	1.380	13.97	10.45	55.68	7.58
4	Nos. 088 and 089	8,200-watt	1.026	1.868	3.698	0.992	17.17	14.60	61.91	7.74
	"Sunshine"	None	1.208	1.801	3.548	1.202	13.29	9.44	39.02	6.30

TABLE III

CARBOHYDRATE COMPOSITION OF BILOXI SOYBEANS GROWN FOR FOUR WEEKS ON 16-HOUR PHOTOPERIODS AT 70° F. WITH VARIOUS RADIATION SOURCES. PLANTS HARVESTED AFTER AN 8-HOUR DARK PERIOD

EXPT. NO.	SOURCES OF RADIATION		PERCENTAGE DRY WEIGHT						MILLIGRAMS PER PLANT					
	CARBONS BURNED IN ARC LAMP	TUNGSTEN-FILAMENT LAMPS	REDUCING SUGARS		SUCROSE		STARCH		REDUCING SUGARS		SUCROSE		STARCH	
			LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
			%	%	%	%	%	%	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	"Sunshine"	8,200-watt	3.564	3.850	0.636	0.839	4.251	1.799	49.14	23.90	8.77	5.21	58.61	11.17
	"Sunshine" upper No. 025 Lower	None	2.647	3.173	0.467	0.577	2.511	1.557	30.54	14.68	5.39	2.67	28.97	7.20
2	"Sunshine"	None	2.194	2.746	0.352	0.780	3.268	1.783	21.19	11.35	3.40	3.23	31.57	7.37
	"Sunshine" upper No. 025 Lower	8,200-watt	2.884	2.622	0.722	0.903	3.361	1.570	44.43	18.14	11.12	6.25	51.78	10.86
3	"Sunshine"	8,200-watt	2.919	3.305	0.924	0.828	3.129	1.532	48.16	25.14	15.25	6.30	51.63	11.66
	Nos. 088 and 089	None	2.499	2.839	0.589	0.635	2.335	1.568	31.10	15.58	7.33	3.48	29.06	8.61
4	Nos. 088 and 089	8,200-watt	4.242	3.516	0.632	0.974	4.963	1.452	71.02	27.47	10.58	7.61	83.09	11.34
	"Sunshine"	None	3.137	3.217	0.532	0.727	2.766	1.133	34.50	16.86	5.85	3.81	30.42	5.94

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The carbohydrate composition of the plants gives a critical measure of the effect of the different radiation sources on photosynthesis. The plants were sampled at the end of a dark period and thus only the carbohydrates remaining were measured.

The percentage of reducing sugars in the leaves was greater in all of the experiments when arc irradiation was supplemented with incandescent irradiation (table III). In experiment 1 all of the carbohydrate fractions were greater in the plants grown with "Sunshine" carbons plus supplemental incandescent radiation than in those grown with the 025 experimental carbon. When the radiation from the 025-"Sunshine" combination of carbons was supplemented with incandescent radiation the amount of carbohydrates in the leaves was increased. However, these amounts were less than those of the plants grown with radiation from the combination of "Sunshine" carbons and incandescent.

In experiment 3 (table III), the carbohydrate content of the plants grown with radiation from the 088 and 089 carbon-arc was less than those grown with the incandescent-supplemented "Sunshine" carbon-arc, and was only slightly greater than those grown in experiment 1 with the radiation from the "Sunshine" upper and 025 lower carbon combination. When the radiation from the 088 and 089 carbons was supplemented with incandescent the residual starch content of the leaves was the highest of any of the experimental lots.

Discussion

The addition of a relatively small amount of incandescent light to the carbon arc resulted in plants with slightly longer internodes, more dry weight, and greater carbohydrate accumulation. It was thought that this was due to a more favorable spectral distribution of the combination since the arc radiation was high in blue and relatively low in red and the incandescent was high in red and very low in blue. However, the dry weight accumulation and the carbohydrate content of soybean plants grown with radiation from carbons cored to emit more red than the combination of "Sunshine" carbons plus incandescent were less in every case. Therefore, the incandescent-filament lamps contributed something more to the production of dry weight than might have been expected from photosynthesis due to the additional red radiation. The incandescent lamps contribute about 28 per cent. of the total radiant energy in each room. However, only 10 per cent. of the incandescent lamp energy is in the visible spectrum. Therefore, the plant responses noted could have been due to (a) increase in the internal temperature of the leaves by infrared radiation, (b) spatial distribution within the room, (c) increased total energy in the visible, or (d) some peculiarity of the spectral distribution of the incandescent radiation on regulatory mechanisms other than photosynthesis.

Data on possible changes of internal leaf temperature resulting from direct radiation were obtained from a thermocouple placed at the under

side of a leaf of a plant growing near the center of the bench. The thermocouple was shielded from air currents by the sides of the leaf which were folded down and fastened with a clip. Under these conditions the temperature at the thermocouple was raised 6.0° F above room temperature when the arc alone was burning, 3.0° F when only the incandescent lamps were on, and 9.0° F when the two sources were burning. Spatial distribution of the lamps may have been partly responsible for the greatly improved plant growth. The carbon-arc radiation was applied from a point source in the center of the room and this undoubtedly resulted in considerable shading of certain leaves. The incandescent lamps were spaced at 8 points more directly above the plants, and it is quite possible that radiation from them could have reached leaves that otherwise would have been shaded. The simple matter of increasing the total irradiance by the addition of 160 foot-candles of incandescent irradiation undoubtedly also contributed to the increase in carbohydrates. It seems improbable, however, that this could be the full explanation because less than a 10 per cent. increase in irradiance resulted in a far greater increase in carbohydrate content. The effect of possible peculiarities of the spectral distribution on growth must be recognized. It is known for example that certain spectral regions are more effective than others in influencing leaf size and shape, length of internodes, and probably other morphological characters.

Many investigators have grown plants with artificial sources of irradiation with varying degrees of success. However, with the exception of MITCHELL (**7**) none has employed the newer types of carbon-arc lamps available. These lamps are designed to hold two pairs of carbons but the arc burns between only one pair at a time. As the carbons of one pair burn back, the arc shifts to the other pair without interruption. This occurs at 20- to 30-minute intervals. The upper carbons are $\frac{7}{8}'' \times 12''$ and the lower carbons $\frac{1}{2}'' \times 12''$. These sizes are used in order to maintain uniform burning rate for both upper and lower carbons so that the arc position remains unchanged throughout the burning period. Operation of the lamps is fully automatic except for carbon changes, which are necessary every eight or nine hours. The radiant energy from these lamps is derived principally from the flame. With this type of arc a part of the carbon can be replaced with chemical compounds capable of radiating efficiently when in a highly heated gaseous form. These compounds vaporize with the carbon and diffuse throughout the flame of the arc, making it luminescent. Since the whole flame is made luminous, the radiation source is one of large area.

The radiation emitted by the flame arc consists chiefly of the characteristic line spectra of the elements volatilized into the flame from the carbon and the coring material, and of the band spectra of the compounds formed. Rare earth metals of the cerium group are used to core the so-called "Sunshine" carbons. This property of being able to change the spectral distribution of the radiation by introducing different chemicals into the coring

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material gives the flame-arc some flexibility for testing the effect of light quality on plant growth. The "Sunshine" carbons produce strong bands of radiation extending from the infrared through the visible and ultraviolet to approximately 2800 A, with a distinct maximum in the blue-violet region. Chlorophyll has a maximum absorption within this band of high energy. Incandescent tungsten sources are very low in blue radiation, and the blue lines of the H-1 mercury arc which coincide with chlorophyll absorption bands account for less than half of the total visible energy. Thus the carbon arc is one of the best artificial sources for intense irradiation in the blue region of chlorophyll absorption.

Several investigators have concerned themselves with spectral balance in radiation sources suitable for growing plants. JOHNSTON (**6**) grew tomato plants in different proportions of red and blue radiation and found that good growth resulted when 14 to 51 per cent. of the total radiation came from high-pressure mercury lamps. DASTUR and SOLOMON (**3**) showed that the carbohydrate content of leaves of several plants was greater when they were irradiated by a carbon-arc than when irradiated with gas-filled incandescent lamps. They also exposed leaves to a "mixed" radiation which was formed by two beams from a single incandescent source. One beam was filtered through a copper sulphate filter which limited the wave-length band to the region 4200 A to 4720 A. The two beams were recombined in the proportion 1 : 1 on an irradiance basis. The carbohydrate content of leaves grown in this "mixed" radiation was greater than that of the plants in the incandescent radiation alone and less than that of leaves exposed to the arc. Greatest photosynthetic activity occurred with the source richest in blue-violet radiation.

In the earlier attempt to grow plants under controlled environment, incandescent lamps were used as the radiation source (**4, 1**). Recently WITHROW and WITHROW (**10**) have compared the radiation from incandescent, fluorescent, and mercury-arc sources on growth of aster, spinach, soybean, and tomato. The three sources were tested alone and the mercury and incandescent in combination. In comparing the sources on an equal power consumption basis, aster produced more dry weight with incandescent and spinach more with fluorescent radiation. Plants grown with mercury-arc source were very poor. The irradiance as measured by a Weston photronic cell corrected to spectral sensitivity of the human eye was 700 foot-candles for the incandescent and 1200 foot-candles for the other two sources. This method of comparison, while interesting from an engineering standpoint, has less utility for comparison of growth responses of plants in different qualities of light than methods that attempt to maintain equal irradiance in the visible.

In another series of experiments WITHROW and WITHROW (**10**) compared the growth of spinach, soybean, and tomato when grown with fluorescent lamps and with incandescent and mercury-arc combined in three different proportions. They report that the greatest production of dry mat-

ter for all species occurred under the incandescent plus low-mercury combination. However, for soybeans grown at 25° C the differences were slight, and since the age of plant was not reported it was impossible to compare their results with others. As the authors show in other experiments, the mercury-arc lamp has a very low efficiency for plant growth due to the fact that nearly two-thirds of the total visible radiant energy of the H-1 mercury arc does not coincide with chlorophyll absorption bands. Therefore, in the experiments reported by WITHROW and WITHROW (**10**) in which an incandescent source was supplemented with a mercury-arc and the radiation held constant at 800 foot-candles, the energy that was useful for assimilation was decreased as the percentage of mercury-arc irradiance was increased; and their data on dry weight accumulation substantiate this point. Their data show that the incandescent source produced more fresh and dry weight than any other source tested.

Our work has shown that a carbon-arc burning "Sunshine" carbons supplemented with incandescent lamps produces a source of radiation that will grow Biloxi soybeans comparable, and in many instances superior, to those grown in a greenhouse. In a series of experiments not reported in this paper soybeans that were 21 days from seeding and grown on 16-hour photoperiods at 70° F averaged 30 cm. in height and 2.23 gm. of dry weight per plant. This is to be compared with greenhouse plants of the same age grown during September that averaged 34 cm. in height and 0.98 gm. of dry weight per plant. WITHROW and WITHROW (**10**) grew Biloxi soybeans at 68° F for 24 days with 18-hour photoperiods of incandescent radiation, and the average height was 36.4 cm. and the average dry weight of tops 1.36 gm. Thus the carbon arc supplemented with incandescent radiation and burning two hours less each day produced a plant that was 6.4 cm. shorter and contained 64 per cent. more dry weight in three days' less time.

All of the available sources of artificial radiation have objectionable features when they are used for plant growth. The important criteria in selecting a source are high intensity and a spectral distribution approaching solar radiation. Fluorescent lamps can be manufactured to give good spectral distribution for photosynthetic activity; however, their low intensity can be a limiting factor with some plants. High-intensity carbon-arc lamps produce a spectral distribution which approaches solar radiation, and the irradiance at a convenient working distance is about 2000 foot-candles. This has proven to be adequate to grow soybean plants to maturity. Somewhat superior results are obtained, however, if the arc is supplemented with some incandescent radiation.

Summary

The growth and composition of soybean plants produced in controlled environment rooms with a carbon-arc lamp as the principal source of radiation have been determined. The arc lamp was used alone with several

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types of carbons, or was supplemented with a small amount of incandescent radiation.

The carbohydrate content of leaves and stems of soybeans was greatly increased by supplementing the radiation from "Sunshine" carbons with incandescent-filament radiation. Experimental carbons cored to simulate the spectral distribution of the combined arc and incandescent source failed to produce as much dry weight, protein, or carbohydrate as when the arc was supplemented with incandescent radiation.

The merits of carbon-arc as a source of radiant energy for plant growth are discussed and compared with other artificial sources. The conclusion is reached that radiation from a carbon-arc supplemented with that from incandescent-filament lamps is superior to any other type for growing many kinds of plants.

PLANT INDUSTRY STATION
BELTSVILLE, MARYLAND

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GROWTH REGULATION BY ORGANIC COMPOUNDS

R. M. Muir, C. H. Hansch and A. H. Gallup

(WITH THREE FIGURES)

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Introduction

A large and diverse group of organic compounds is reported to "regulate" plant growth. Many of the determinations of growth regulation, however, do not measure the accelerating effects on growth but the inhibition of growth or toxicity effects. Such evidence does not suffice to identify the substances as growth regulators which may either accelerate or inhibit the growth process depending upon their concentration.

Many techniques exist for the quantitative determination of the acceleration of plant growth by organic compounds (**4, 9, 10, 11, 16, 17,**). Of these the straight growth of Avena coleoptile sections has distinct advantages. This test gives quantitative data on growth response, it enables the study of accessory factors involved in the growth process, it is sensitive to the effects of substances which do not cause an Avena curvature response, and it does not depend on the complicated series of reactions of the pea test.

This study was made to determine the similarity of the effects of several organic compounds and the plant auxin, indoleacetic acid (**20**), on the straight growth of Avena coleoptile sections. Most of the compounds have been studied by other investigators but their results cannot be correlated satisfactorily because of the different techniques employed in the measurement of the growth effects.

Materials and methods

Test plants were grown according to the procedure described by Bonner (**4**). After 96 hours the apical 2 mm. of the coleoptile were removed and two hours later a single section 3 mm. in length was cut from the apex with the primary leaf included within the coleoptile. The cutter used in these experiments gave sections with a standard deviation of initial length of $\pm$ 0.03 mm. Fifteen sections were floated on the surface of 25 ml. of the solution in a Petri dish and placed in an incubator at 28° C for 24 hours. The sections were not mounted on glass rod or combs (**4, 10**) as curvature during the growth period occurred very infrequently. Growth measurements were made with an ocular micrometer mounted in a binocular dissecting microscope at a magnification of eight diameters. Most of the compounds which were tested were freshly prepared. A few were the products of commercial laboratories recrystallized several times before use. Melting points of all compounds were determined to check their purity.

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Experimental results

The residual auxin in the coleoptile section allows for an elongation of the section floating on distilled water. This elongation varies considerably depending on the conditions under which the test plants are grown. In these experiments it was usually 8 per cent. of the original length. With each experiment the growth in distilled water was determined and the data are expressed as the percentage elongation of sections in a given solution of the chemical minus the percentage elongation in distilled water. Differences of 1 or 2 per cent. are recorded as zero. At high concentrations of the organic compounds an inhibitory effect on elongation becomes apparent

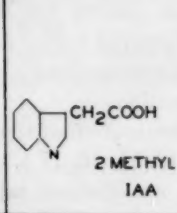
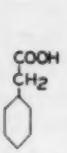
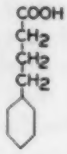
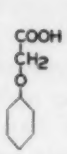
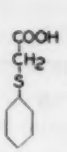
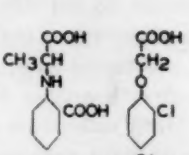
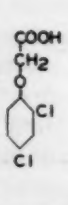
MOLAR CONC. $\times 10^{-4}$	 2 METHYL IAA						
10.0	−12						
5.0	− 6	−12	− 4	−23	−11	−14	−10	−13
2.0	+23	+10	+25	−11	+ 6	− 3	+15	− 8
1.0	+38	+32	+30	+13	+10	+15	+16	+10
0.5	+36	+29	+31	+16	+ 6	+ 9	+ 8	+28
0.1	+36	+16	+19	+13	+ 3	0	0	+30
0.05	+29	+ 8	+14	+ 8	0			+31
0.01	+25	0	+ 8		0		0	+24
0.005		0				0	0	+21
0.001	0				0			+ 7

FIG. 1. Comparison of the effects on cell elongation of several organic compounds and the plant auxin (indoleacetic acid). Values are the differences between percentage elongation in solutions of the chemicals and percentage elongation in water.

(negative values in figures). Death of the tissues generally occurs at a concentration of 2×10^{-4}M or higher, and a marked shrinkage in length results from a loss of turgor. The amount of food material available and the amount of growth regulating substance together determine the maximum elongation (**10**). Thus the relative activity of different chemicals is indicated by the molecular concentration sufficient to bring about maximum elongation as well as by the relative elongation occurring at any particular concentration of the chemicals.

Many recent investigations present evidence of the growth regulating properties of the selective herbicide, 2,4-dichlorophenoxyacetic acid (re-

ferred to as 2,4-D hereafter). AKAMINE (1) reports the initiation of root development on cotyledons by 2,4-D, ZIMMERMAN (22) reports it to be active in causing stem and petiole curvature and parthenocarpy, and FULTS and PAYNE (5) report it active in the pea test. However, in the Avena deseeded test 2,4-D has only 0.1 per cent. of the activity of indoleacetic acid (IAA) according to AVERY, BURGER and SHALUCHA (2). The data in figure 1 show that 2,4-D is a growth regulating substance of equal or greater effect on cell elgonation than IAA!

Tests of compounds related to 2,4-D were made to determine, if possible,

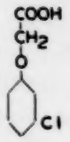
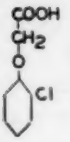
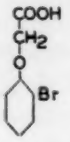
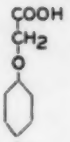
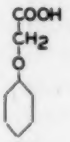
MOLAR CONC. X 10^{-4}					
5,0	−16	− 6	−11	−13	−14
3,0	−11	− 4	− 8		
2,0	− 6	+11	0	− 8	−11
1,0	+21	+13	+20	+ 9	+13
0,5	+25	+ 9	+ 9	+19	+21
0,1	+11	+ 3	0	+14	+14
0,05	+ 9	0	0	+13	
0,01	+ 4		0	+ 9	+ 3
0,005	0		0	0	
0,001	0		0	0	

FIG. 2. Effect of monohalogen derivatives of phenoxyacetic acid on cell elongation. Values are the differences between percentage elongation in solutions of the chemicals and percentage elongation in water.

the structural features responsible for its activity. Phenylacetic acid (PAA) was reported to have little activity in the regulation of cell elongation until THIMANN and SCHNEIDER (13) found it to have a maximum elongation effect which was about half that of IAA. BONNER (3) reports that if corrections for greater dissociation are made, the activity of PAA approaches that of IAA. The results of tests given in figure 1 show that PAA has an activity which approaches that of IAA at high concentrations but not at low concentrations. THIMANN and SCHNEIDER (13) also found that γ-phenylbutyric acid (PBA) directly accelerated cell elongation although

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other investigators had ruled it inactive. In the tests reported here the elongation induced by PBA is approximately half that induced by PAA. The data for phenoxyacetic acid (POAA), S-phenylglycolic acid, and o-(carboxyphenylamino) propionic acid in which an oxygen atom, a sulfur atom and a nitrogen atom respectively are introduced between the acetic acid group and the benzene ring show that they have less activity than PBA in which two carbon atoms are introduced similarly. The activity of 2,4-D is not a consequence of the introduced oxygen atom alone.

The contributions of the substituted halogen atoms to the growth activity

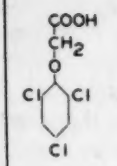
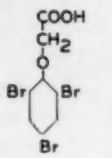
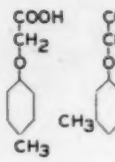
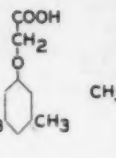
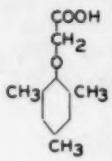
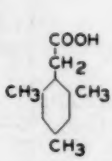
MOLAR CONC. X 10^{-4}						
5.0			−12	−11	−12	−10
2.0			+ 8	− 5	− 4	
1.0	−11	−11	+17	+ 9	+ 9	+ 3
0.5	+ 4	− 3	+ 6	+11	+ 7	+ 3
0.1	0	0	0	+ 5	+ 4	0
0.05	0	0			0	0
0.01	0	0	0	+ 3	0	0
0.001	0	0				

FIG. 3. Comparison of the effects of several organic compounds on cell elongation. Values are the differences between percentage elongation in solutions of the chemicals and percentage elongation in water.

of 2,4-D were investigated by testing first, the effects of a single chlorine or bromine atom in the ortho, meta or para positions of POAA and second, the effects of substitution of chlorine or bromine in the 2,4 and 6 positions. The data given in figure 2 indicate that the substitution of a single halogen atom in the ortho, meta or para position increases the growth activity of the POAA structure. Substitution in the para or meta position has a greater effect than substitution in the ortho position but does not equal the effect of 2,4-D which has chlorine atoms at both the ortho and para positions.

In figure 3 the results of tests of the 2,4,6-trichlorophenoxyacetic acid

and the similar bromine compound show them to be inactive, the chlorine in the 6 position nullifies both the 2,4-D activity and that associated with the POAA structure. Similar effects have been noted by ZIMMERMAN (**21**) and SYNERHOLM and ZIMMERMAN (**11**) in their growth tests. They also report that 2,6-dichlorophenoxyacetic acid, 2,3,4,6-tetrachlorophenoxyacetic acid and the pentachlorophenoxyacetic acid are all inactive but the 2,3,4- and 2,4,5-trichlorophenoxyacetic acids are active. Incomplete data are available which indicate that 2,4,5-trichlorophenoxyacetic acid has an effect on cell elongation which approaches that of 2,4-D.

It was of interest to determine the effects of substituted methyl groups and compare them with the substituted halogens. The data in figure 3 show the growth activities of para methylphenoxyacetic acid, 3,5-dimethyl- and 2,4,6-trimethylphenoxyacetic acids which differ very little from that of POAA. Substitution of a methyl group does not have the augmenting effect on growth activity that substitution of a halogen has, nor is the activity of the POAA structure completely nullified by the substitution of methyl groups at the 2,4 and 6 positions as was the case for the similar halogen substituted compounds. Substitution of methyl groups at the 2,4 and 6 positions of PAA however, does nullify the growth activity. Similar diminution of the growth activity of IAA occurs upon substitution of a methyl group in the 2 position (fig. 1). This effect was reported by KÖGL and KOSTERMANS (**8**) who found also that substitution of an ethyl group at this position caused complete loss of growth activity.

Discussion

KOEPFLI, THIMANN and WENT (**7**) propose that an active growth regulator has an unsaturated ring system with a side chain carrying a carboxyl group at least one carbon atom removed from the ring. WENT (**19**) states that the side chain must be adjacent to the ring double bond. The chemical constitution of the phenoxyacetic acids meets all of these structural requirements for plant growth activity. Blocking of both positions ortho to the side chain carrying the carboxyl group, however, destroys the growth promoting activity. Therefore, in addition to the other structural requirements, the active phenoxyacetic acid compound must have a free, or potentially free ortho position.

In addition to these structural requirements, other properties have been used to account for the differences in growth regulating activity. According to BONNER (**3**) differences in the degree of ionization of the acids may be used to explain relative growth promoting activities of similar compounds. Attempts to apply this theory to some of the compounds tested in this investigation have been unsuccessful. The dissociation constants of the acids as reported by HAYES and BRANCH (**6**) are given in table I. Phenoxyacetic acid is less ionized than any of the halogen acids, yet the latter all have greater growth activity. Similarly the ortho and para halogen compounds have about the same ionization constants yet the para compounds are much more active. VELDSTRA (**15**) shows that differences in

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dissociation of the acids derived from naphthalene also do not explain their relative growth activities.

VELDSTRA (**14**) proposes that the properties of active growth regulators are associated with an adsorption of the active substance at a boundary surface. A more common interpretation of the activity of growth regulators involves a reaction with a substrate (**12, 18**). The lack of activity of compounds substituted in both ortho positions is best explained by the latter theory. Thus growth regulators may be considered as reacting with a substrate at two points, first by means of the carboxyl group, and second at a position ortho to the attachment of the carboxyl group. This would explain the striking lack of growth activity of 2,4,6-trichloro- and tribromophenoxyacetic acids compared to the great activity of 2,4-D and 2,4,5-trichlorophenoxyacetic acid. Substantiating evidence may be adduced from the lack of activity of 2,4,6-trimethylphenylacetic acid with substituents in both ortho positions whereas phenylacetic acid is almost as active as IAA. Since

TABLE I

DISSOCIATION CONSTANTS OF COMPOUNDS TESTED FOR GROWTH ACTIVITY

COMPOUND	K
Phenylacetic acid	4.88×10^{-5}
Phenoxyacetic acid	6.75×10^{-4}
p-chlorophenoxyacetic acid	7.89×10^{-4}
p-bromophenoxyacetic acid	7.37×10^{-4}
p-methylphenoxyacetic acid	6.09×10^{-4}
o-chlorophenoxyacetic acid	8.90×10^{-4}
o-bromophenoxyacetic acid	7.53×10^{-4}
m-chlorophenoxyacetic acid	8.51×10^{-4}

the activity of 2,4,6-trimethylphenoxyacetic acid is not less than that of POAA, it is possible that the reaction with the substrate may take place through a methyl group or by removal of a methyl group. Experiments are in progress to test these hypotheses more rigorously.

Summary

IAA and 2,4-D have equal growth activity as measured by cell elongation. Other substituted phenoxyacetic acids have growth activities which vary from small values to values equalling that of 2,4-D. The growth activity of 2,4-D and other phenoxyacetic acids can be related to chemical structure by the supposition that the position on the benzene ring adjacent to the point of attachment of the side chain is directly involved in the growth reaction.

BOTANY DEPARTMENT
STATE UNIVERSITY OF IOWA
IOWA CITY, IOWA
AND
CHEMISTRY DEPARTMENT
POMONA COLLEGE
CLAREMONT, CALIFORNIA

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THE NITROGEN COMPOSITION OF CEREAL GRASSES. I. PRINCIPAL NITROGEN FRACTIONS[1]

ROBERT MACVICAR

Received August 30, 1948

The availability of winter cereal grasses in the Southwest during the late fall and early spring has focused attention on these plants as a source of feed for livestock and poultry. Extensive investigations have been conducted at this station by STATEN (**9**) during the last few years to determine the total yield of grass for grazing livestock and the effect of clipping on grain yields. Most of the varieties tested have been analyzed for proximate constituents by HELLER (**4**). Selected varieties have also been analyzed for carotene and certain vitamins of the B-complex by MOYER, *et al.* (**6**).

One of the observations made during these studies was the high nitrogen content of these cereal grasses when clipped at grazing height. A crude protein content in the range of 20–40 per cent. on a dry weight basis ($N \times 6.25$) was common. In view of these high values, it became of interest to ascertain the distribution of the principal nitrogenous fractions. Little information on the nitrogen composition of these plants was available in the literature. Furthermore, much of the data had been collected using more mature plants. MILLER (**5**) in an investigation of wheat showed in two varieties that the non-protein nitrogen of the leaves and stems constituted approximately 25 per cent. of the total, and occasionally approached 40 per cent. What portion of the non-protein nitrogen was present in the form of amino acids was not determined. The extensive investigations of CHIBNALL and his students (**1**) on the pasture plants and grasses of England and Australia have shown that only a portion of the nitrogenous constituents were of such a type as to be coagulable by acid and heat. CULTERA (**3**) in studies on the nitrogen metabolism of wheat and oats had reported that approximately one-third of the total nitrogenous compounds are not precipitated with trichloroacetic acid. In view of these data, five representative species of cereal grasses and annual rye grass have been sampled throughout the fall and spring growing seasons for two consecutive years and analyzed for their principal nitrogenous constituents. The present communication presents the results of these determinations.

Materials and methods

COLLECTION OF MATERIAL.—Five representative cereal grasses, rye (*Secale cereale*, L., var. Balbo), oats (*Avena sativa*, L., var. Wintok), barley (*Hordeum vulgare*, L., var. Michigan Winter), hard wheat (*Triticum*

[1] Published with the approval of the Director, Oklahoma Agricultural Experiment Station.

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aestivum, L., var. Tenmarq), soft wheat (*Triticum aestivum*, L., var. Clarkan) and annual ryegrass (*Lolium multiflorum*, Lam.) were used in this study. The plants were produced on well fertilized Kirkland Silt Loam near Stillwater, Oklahoma during the 1946–47 and 1947–48 grazing seasons. The experimental plot design and methods used in the collection of samples have been previously described in detail (**9**). Plants were clipped whenever they reached grazing height (3–4 inches), the clippings thoroughly mixed, and representative samples selected for analysis.

Extraction Procedures.—After trials of several widely differing techniques for the preparation of extracts containing a major portion of the nitrogenous constituents of the plants, the following procedure was adopted: 50 grams of thoroughly mixed plant tissue was selected at random and placed in the cup of the Waring Blendor. A sufficient quantity of pH 6.5, 0.02 M phosphate buffer was added to permit ready homogenization, and the blendor operated at high speed until the tissues were reduced to a uniform mass. A few drops of caprylic alcohol were used to prevent foaming. The tissue extract was then filtered through a layer of coarse cheese cloth, which retained the fibrous material and permitted a major portion of the chloroplasts to pass through. The material remaining on the filter was successively extracted in the blendor until the extracts were only faintly green in color. The material remaining after the final extraction was composed almost wholly of the structural material of the leaf. Analysis showed that in most cases 93–95 per cent. of the total nitrogen of the leaf was extracted in this manner. Microscopic examination revealed very few unruptured cells; it, therefore, appears likely that this remaining nitrogen represented occluded chloroplasts. The extracts were made to volume with distilled water and aliquots taken immediately for analysis, thus minimizing enzymatic changes. The remainder of the sample was covered with a film of toluene and stored under refrigeration until the determinations performed on the untreated extract were completed, within 12–24 hours.

Fractionation Procedures.—The fractionation procedure followed was essentially as follows: Total nitrogen; nitrogen content of the extract as determined by Kjeldahl digestion, hence did not include all the nitrate; non-protein nitrogen, that portion not precipitated by 5 per cent. trichloroacetic acid; non-protein alpha-amino nitrogen, determined on the TCA filtrate by the nitrous acid procedure of Van Slyke; non-protein nitrogen precipitated by Neuberg's reagent, that portion of the TCA filtrate precipitated by mercuric acetate at pH 8 from 30 per cent. ethanol solution; Neuberg filtrate, nitrogen present in filtrate of previous treatment.

Determination of Nitrogen.—The nitrogen in the preceding fractions was determined by a semi-micro modification of the Kjeldahl procedure using copper and selenium catalysis. Since reduction did not precede digestion, these values do not include all the nitrate. Ammonia and amide nitrogen were determined in the original extract by the method of Pucher,

et al. (7) with minor modifications; aeration rather than distillation was employed to remove the ammonia which was determined titrimetrically. The amide nitrogen was hydrolyzed with acid, and the ammonia formed was removed by aeration. The nitrate nitrogen remaining in the extract after the determination of ammonia and amide nitrogen was reduced to ammonia with Devarda's alloy and removed by aeration.

MOISTURE.—The moisture content of the tissue was determined by drying representative samples to constant weight in a forced-ventilation constant-temperature oven at 60° C.

Results and discussion

The average composition of these grasses for successive samplings during the 1946–47 and 1947–48 growing seasons, together with the pertinent statistical analysis by the method of variance (SNEDECOR, 8), is presented in table I.

TABLE I

SUMMARY OF NITROGEN COMPOSITION ON DRY WEIGHT BASIS OF REPRESENTATIVE VARIETIES OF CEREAL GRASS CLIPPINGS

NITROGEN FRACTION	VARIETY						AVERAGE ALL VARIETIES
	MICHIGAN WINTER BARLEY	WINTOK OATS	BALBO RYE	TENMARQ WHEAT	CLARKAN WHEAT	ANNUAL RYEGRASS	
	mg/gm	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
Total	32.8	34.6	32.6	30.6	33.6	31.9	32.7
Non-protein N	10.4	11.0	12.3	10.5	10.6	7.5	10.4
Non-protein alpha-amino N	6.2	6.7	6.2	5.5	6.2	5.7	6.1
Neuberg Filtrate N	2.1	1.8	2.2	2.0	2.3	1.8	2.0
Neuberg Ppt. N	7.8	9.4	9.9	8.1	8.3	5.8	8.2
Ammonia	1.87	1.82	2.36	1.76	2.14	2.29	2.04
Nitrate N	1.09	0.89	1.02	0.63	0.84	1.62	0.99

Mean Squares from Analysis of Variance

Source of Variation	Degrees of Freedom	Total N	NPN	NPN α-amino N	Neuberg Filtrate N	Neuberg PPt. N	NH_3 + Amide N	Nitrate N
Date of Sampling	8	120.1**	11.2	6.5**	1.3**	9.6**	0.35	2.07**
Variety	5	16.9	19.1*	1.7	0.3	18.8**	0.59**	1.04**
Error	39	20.2	7.2	1.2	0.3	2.3	0.18	0.19

It will be seen from the statistical analysis that the most pronounced variation observed in the nitrogen composition of these grasses was with the time of sampling. Considerable variation was found for all fractions determined from one sampling date to another; these differences were found to be highly significant for all fractions except NPN and ammonia

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plus amide nitrogen. In general, the total nitrogen content was elevated in samples collected during periods favorable to rapid growth. Nitrate nitrogen was particularly variable, in some instances only traces being found. During periods of reduced soil temperature in the late fall and early spring, the nitrate content of the plants was usually lowered. This diminution was possibly due to reduced microbiological activity in the soil during cooler weather with concommitant reduction in available nitrates.

Little difference was found in the average total nitrogen content of the different varieties. Varietal differences did occur in the NPN fraction which were significant at the five per cent. level and in the NH_3 plus amide fraction and the nitrate fraction with significance at the 1 per cent. level. The difference in NPN was due to the lower content of annual ryegrass—differences between the other cereals not being demonstrable. Varietal differences in the minor nitrogen fractions were highly significant; in the case of nitrate this was also due to the higher nitrate content of annual ryegrass.

Among the true cereal grasses approximately two-thirds of the total nitrogen was present in a form precipitable with 5 per cent. trichloroacetic acid. These data are in essential accord with those of CULTERA (**3**) who examined the nitrogenous fractions of wheat and oats using a similar technique.

The fact that such a large portion of the nitrogen present in the TCA soluble fraction was precipitated with Neuberg's reagent suggests that this nitrogen is present principally as polypeptides and amino acids. This reagent precipitates a wide variety of nitrogen-containing compounds including peptides and amino acids. The alpha-amino nitrogen as measured by reaction with nitrous acid accounted for roughly one-half to two-thirds of the total in the non-protein fraction. The alpha-amino nitrogen accounted for nearly all of that present in the Neuberg precipitate in some varieties and only for about 60 per cent. in others. Such varietal differences, if such indeed exist, might be due to incomplete precipitation by the relatively non-specific reaction with alkaline mercuric acetate. Only in an occasional instance did the total of ammonia plus amide nitrogen account for more than 5 per cent. of the total. This would suggest that practically all of the nitrogen not in the form of true protein is present, in the main, as amino acids and their condensation products. The variable nitrate content has been previously mentioned.

Comparison of these data with those of CRUICKSHANK and WOOD (**2**) is of interest. These workers employed Algerian oats (*Avena sterilis* L.) in a study of nitrogen metabolism in starving leaves. Freshly harvested samples contained somewhat less total nitrogen on a dry weight basis than did our material; this may have been due to the greater age of the plants employed by them. A total nitrogen content somewhat less than the minimal values found by us was also observed by VINCENT and co-workers (**10**)

in barley. CRUICKSHANK and WOOD (**2**) also found a principal portion of the total nitrogen to be protein. Values for ammonia and nitrate nitrogen correspond roughly to the minimum values obtained by us. They report only traces of glutamine and asparagine at the time of harvest; considerable loss in amide fraction, however, might well have been entailed by the method of drying the samples at 90° C. CULTERA (**3**) found wheat and oats grown in pot culture to contain ammonia and amide nitrogen levels approximately one half the average found by us; their values, however, fall well within the range observed in this study.

These data indicate that these cereal grasses would have a relatively high nutritional value, as measured by chemical analyses, for a source of protein. Since such a major portion of the nitrogen is present in the form of protein, peptides or free amino acids, these grasses should be equally valuable for non-ruminants and poultry. Studies on the amino acid composition of the protein of these grasses are now in progress.

Summary

The principal nitrogen fractions of cereal grasses periodically clipped whenever grazing height was reached have been determined for five representative species of cereals and annual ryegrass. The average composition for all species was as follows: total nitrogen, 32.7; non-protein nitrogen, 10.4; non-protein alpha-amino nitrogen, 6.1; non-protein nitrogen precipitable by Neuberg's reagent, 8.2; non-protein nitrogen in Neuberg filtrate, 2.0; ammonia plus amide nitrogen, 2.04; and nitrate nitrogen, 0.99 milligrams per gram of dry leaf tissue. A significantly greater part of the nitrogen of annual ryegrass was present in a form precipitable by trichloroacetic acid (protein nitrogen). About 60 per cent. of the non-protein nitrogen fraction was present in the form of amino acids. Significant varietal differences were found in the ammonia plus amide and nitrate nitrogen content; these composed only a small portion of the total nitrogen. Because of the relatively high nitrogen content, most of which is present in combined form, these grasses provide a valuable forage crop.

Appreciation is expressed to Professors Carl Marshall of the Statistical Laboratory for aid in the statistical analyses and H. W. Staten and V. B. Hawk for providing experimental material.

DEPARTMENT OF AGRICULTURAL CHEMISTRY RESEARCH
OKLAHOMA AGRICULTURAL EXPERIMENT STATION
STILLWATER, OKLAHOMA

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ORGANIC ACIDS OF THE COTTON PLANT[1]

DAVID R. ERGLE AND FRANK M. EATON

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The quantities of the organic acids occurring in the leaves of cotton have been found to be comparable in amount with the proteins and often several times greater than the sugars plus starch. As in other plants (**2, 10, 18, 22**), such a mass of little understood material incites interest as to its function and lability. It has been suggested than an organic acid may be an intermediary in the photosynthetic process and also that these acids may be of significance in the initiation of reproduction. In either such role it seems doubtful that any great part of a mass constituting 10 or 20 per cent. of the dry weight of the leaves could be very active. Although a nutritional role may not be eliminated it has been common to regard the organic acids as neutralizing or buffering agents. The object of the work back of this paper has been that of gaining some insight into the functional activity of the organic acids of cotton. The behavior of these acids has been studied by means of experiments wherein such factors as moisture supply, mineral nutrition, fruitfulness, and respiration were varied.

Analytical methods

Preparatory to analysis all tissues were dried at 75° C. in a forced-draft oven and ground in a small Wiley mill to pass an 80-mesh screen.

Ash constituents were determined by A.O.A.C. official methods (**1**).

Determination of the various carbohydrate fractions and cellulose was based upon procedures described elsewhere (**6, 7**).

The ether-extraction and subsequent preparation of an aqueous solution of the organic acids from the cotton tissues was accomplished by the method of PUCHER *et al* (**15**). Total organic acids were determined by titrating aliquots of the aqueous solution between the limits of pH 7.8 and pH 2.6, using the method of VAN SLYKE and PALMER (**21**). Additional aliquots of the same solution were analyzed for citric, malic, and oxalic acid, following the procedures of PUCHER *et al* (**16**). The "unidentified" portion of the total organic acids was obtained by subtracting the sum of citric, malic, and oxalic acid from the determined value for total organic acids.

Tests were made for isocitric and *cis*-aconitic acids by the method of KREBS and EGGLESTON (**8**), using an extract of aconitase derived from frozen breast muscle of pigeon. As tested on plants known to contain isocitric and *cis*-aconitic acids the aconitase thus obtained was found to be active.

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Experimental

Distribution and kinds of acids

Of the parts of the cotton plant which have been examined, higher concentrations of organic acids are found, table I, in the leaves than in other vegetative parts. Next in order come the petioles and then the stems and the roots, each of which contains about half as much as the petioles. The flower as a whole contains less organic acid than the petioles, and 13-day old bolls and mature seed kernels contain still less. Organic acids have been found by McCall and Guthrie (9) to constitute 0.84 per cent. of the dry weight of raw cotton fiber.

In all tissues except seed kernels there was less citric than malic acid and only in the flowers did oxalic exceed malic. The "unidentified" acids

TABLE I

Distribution of organic acids in various parts of the cotton plant. Milliequivalents per 100 grams dry tissue

Plant part	Citric	Malic	Oxalic	Unidentified	Total
Leaves	105.6	119.6	11.5	39.2	275.9
Petiole	22.1	70.9	21.6	40.2	154.8
Stem	7.5	27.0	9.8	28.5	72.8
Root	3.4	17.0	14.9	34.8	70.1
Flowers	14.7	17.8	27.3	48.5	108.3
13-day bolls	4.5	18.7	7.3	28.9	59.4
Seed kernels	12.5	11.2	7.8	.....	30.9
Raw cotton fibers*	1.1	7.0	0.1	4.9	13.1

*Average of data by McCall and Guthrie (9).

exceeded malic in stems, roots, 13-day old bolls, and flowers, but not in the leaves, petioles, raw cotton fibers, or seed kernels.

Attempts to identify the organic acids comprising the "unidentified" group were limited to tests for isocitric and *cis*-aconitic acids. In the cotton tissues examined neither of these acids was present in amounts sufficient to be demonstrated by Krebs' (8) micro-method.

Influence of water supply on the citric acid-malic acid equilibrium

The data reported in table II were derived from an experiment with Acala P18-c cotton conducted at Shafter, California in 1946. The plants were grown in plots irrigated weekly which showed no wilting at any time and in plots which were not irrigated after early June. The latter plants wilted severely each afternoon from early July through August and September. At the end of the season the dry plants weighed about one-fifth as much as the plants in the weekly-irrigated plot. The leaf samples were collected on July 16 and 17 and again on August 6 and 7. For each sampling there were two mid-morning and two mid-afternoon collections of

TABLE II

INFLUENCE OF MOISTURE AND FRUITFULNESS ON THE ORGANIC ACIDS OF COTTON LEAVES, STEMS, AND ROOTS (MILLIEQUIVALENTS PER 100 GRAMS DRY WEIGHT), AND ON THE LEVEL OF SUGARS AND SUGARS PLUS STARCHES, AND LEVEL OF NITROGEN

	Organic acids					Moisture	Carbohydrates		Nitrogen	
	Citric	Malic	Oxalic	Unidentified	Total		Sugars	Sugars plus starch	Soluble	Total
Leaves										
Treatment	*m.e.*	*m.e.*	*m.e.*	*m.e.*	*m.e.*	%	%	%	%	%
Wet										
Full load	102.4	90.5	8.8	77.5	279.2	78.6	1.11	2.81	0.63	3.28
2-boll	98.2	97.8	8.3	56.8	261.1	77.9	1.28	3.70	0.57	3.42
Dry										
Full load	44.8	142.7	12.9	67.4	267.8	71.3	.96	2.24	0.78	3.08
2-boll	50.1	143.8	13.2	68.1	275.2	70.5	.97	2.19	0.68	3.09
Mean change as a result of:										
Drought	−52.9	49.1	4.5	0.6	1.4	−7.4	−.23	−1.04	.13	−.26
2-boll	0.5	4.2	−0.1	−10.0	−5.4	−0.7	.09	.42	−.08	.07
Stems										
Treatment										
Wet										
Full load	7.7	30.9	9.1	44.0	91.7	76.8	3.92	5.07	0.33	0.78
2-boll	8.3	34.9	10.8	31.1	85.1	75.9	4.14	5.41	0.32	0.91
Dry										
Full load	5.7	22.3	14.1	46.1	88.2	66.4	7.19	10.43	0.44	0.94
2-boll	6.1	24.2	13.1	38.1	81.5	64.3	6.13	11.89	0.44	1.00
Mean change as a result of:										
Drought	−2.1	−9.7	3.7	4.5	−3.6	−11.0	2.63	5.92	.12	.13
2-boll	0.5	2.9	0.3	−10.4	−6.6	−1.5	−.42	.90	.00	.00
Roots										
Treatment										
Wet										
Full load	3.9	13.4	10.9	36.5	64.7	72.0	3.34	5.73	0.25	0.65
2-boll	3.6	12.2	10.9	33.0	59.7	70.8	3.71	6.81	0.28	0.62
Dry										
Full load	4.7	9.8	15.6	32.4	62.5	62.8	6.59	13.54	0.27	0.70
2-boll	3.1	9.8	13.8	25.3	52.0	58.4	5.05	15.58	0.29	0.72
Mean change as a result of:										
Drought	0.2	−3.0	3.8	−5.9	−5.0	−10.8	2.29	8.29	.02	.08
2-boll	−1.0	−0.6	−0.9	−5.3	−7.8	−2.8	−.58	1.56	.02	.00

three plants from each treatment. The leaves from twelve plants were composited for each treatment sample. The means of the two sets of samples are reported in table II. The leaf samples are from the middle third of the main stalk, the stem samples are the middle third of the main stalk, and the root samples are the tap and large lateral roots as lifted with a shovel.

In the leaves, table II, there was under drought a loss of 52.9 m.e. (per 100 gms. of dry sample) of citric acid and a gain of 49.1 m.e. of malic acid and also a gain of 4.5 m.e. of oxalic acid. In the stems and roots, citric acid was changed little with drought and malic acid was apparently decreased.

The increase in malic and loss of citric acid in the leaves of the dry plants occurred both in plants with a full load of bolls and, in similar pro-

TABLE III

INFLUENCE OF MOISTURE SUPPLY ON THE ORGANIC ACIDS OF THE LEAVES OF TWO VARIETIES OF COTTON. MILLIEQUIVALENTS PER 100 GRAMS DRY WEIGHT. JULY 24, 1946

	Organic acids					Moisture	Carbohydrates		Nitrogen	
	Citric	Malic	Oxalic	Unidentified	Total		Sugars	Sugars plus starch	Soluble	Total
	m.e.	*m.e.*	*m.e.*	*m.e.*	*m.e.*	%	%	%	%	%
Acala P 18-C										
Wet plot	125	109	2	27	263	79	1.41	5.36	.45	3.13
Dry plot	49	205	4	20	278	73	1.58	2.01	.86	3.11
Stoneville 2B										
Wet plot	115	169	6	6	296	78	1.33	4.38	.67	3.11
Dry plot	55	235	11	.....	299	68	1.96	2.18	.79	2.72
Mean change as a result of drought:	−68	81	4	−7	9	−8	.40	−2.78	.27	−.21

portions, in plants maintained with only two bolls each by bud and flower removal. In neither treatment did drought have a significant effect on total organic acids.

Other leaf samples of both Acala P18-c and Stoneville 2B cotton were collected from similarly treated plots on July 24, and the organic acids in these samples were also determined. In this instance, table III, as before in table II, large reductions were found in the concentrations of citric acid in the dry-plot leaves. There were large and roughly equivalent, or greater, gains in malic acid.

The analyses reported in table III were made on one-third of the 110 to 140 leaves (blades only) collected from each treatment. The second and third aliquots—taken leaf by leaf in turn in the order of collection—were placed respectively between wet and dry towels and allowed to stand in the laboratory for 24 hours before being dried in the oven. The data from these

TABLE IV

ORGANIC ACIDS, CARBOHYDRATES, AND NITROGEN IN COTTON LEAVES FROM WET AND DRY PLOTS. ALSO LEAVES FROM SAME COLLECTIONS AFTER STANDING BETWEEN EITHER WET OR DRY TOWELS FOR 24 HOURS. SHAFTER, CALIFORNIA, JULY 24, 1946. ORGANIC ACIDS IN MILLIEQUIVALENTS PER 100 GRAMS DRY WEIGHT. OTHER CONSTITUENTS ON PER CENT. OF DRY WEIGHT

	Organic acids					Moisture	Carbohydrates		Nitrogen	
	Citric	Malic	Oxalic	Unidentified	Total		Sugars	Sugars plus starch	Soluble	Total
	m.e.	*m.e.*	*m.e.*	*m.e.*	*m.e.*	%	%	%	%	%
ACALA P18-C										
Wet plot										
Original	125	109	2	27	263	79	1.41	5.36	.45	3.13
Wet towel	126	94	1	34	255	81	.60	1.33	.57	3.15
Dry towel	108	124	6	4	242	69	1.14	1.23	1.20	3.23
Dry plot										
Original	49	205	4	20	278	73	1.58	2.01	.86	3.11
Wet towel	60	175	8	28	271	76	.35	.55	.92	2.98
Dry towel	50	220	10	.....	275	68	.69	.79	.88	3.14
STONEVILLE 2B										
Wet plot										
Original	115	169	6	6	296	78	1.33	4.38	.67	3.11
Wet towel	129	142	13	33	317	79	.58	1.27	.83	2.89
Dry towel	121	154	10	27	312	66	1.66	2.18	1.12	3.10
Dry plot										
Original	55	235	11	.....	299	68	1.96	2.90	.79	2.72
Wet towel	66	205	8	19	298	72	.98	1.73	.72	2.92
Dry towel	54	239	10	.....	297	61	1.23	1.40	1.06	2.71
MEANS										
Original	86	180	6	14	284	74	1.57	3.66	.69	3.02
Wet towel	95	154	8	29	285	77	.63	1.22	.76	2.99
Dry towel	83	184	9	8	282	66	1.18	1.40	1.07	3.05

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24-hour samples, together with a repetition of the data from the original samples, are reported in table IV.

Comparing first the wet-towel and dry-towel samples, we find a further confirmation of the moisture relation brought out in tables II and III, *i.e.* with the reduction in moisture supply there was a loss of citric acid and a gain in malic acid in each of the paired data of each of the two cotton varieties. The gain in malic averaged 30 m.e. and the loss in citric averaged only 12 m.e. But in these 24-hour samples there was also a loss of 21 m.e. of "unidentified" acid.

The loss of citric acid and gain in malic acid with moisture reduction represents, apparently, a reversible reaction. This is shown when the "original" samples are compared with the "wet towel" samples. All of the paired data between the wet towels show that while there was a gain in citric and a loss in malic acid, the average loss in malic acid was 26 m.e. and the average gain in citric was 9 m.e. But over this 24-hour period there was also a gain of 15 m.e. in the "unidentified" acid.

The foregoing data thus show that whether under conditions of growth and respiration in the field or under conditions of respiration in the laboratory there is a reversible interchange, though necessarily indirect, between citric and malic acids. As moisture is lost the change is toward malic and as it is gained the change is toward citric. The 24-hour results also show that the "unidentified" acids are involved in the reaction, *i.e.* the reaction did not go to completion in 24 hours. Instead, a part of the precursor, or derivative, of the citric acid required to equal the gains, or losses, in malic was apparently still included in the "unidentified" acids at the end of 24 hours.

The conversion of malic to citric acid has been observed previously by others under other conditions, but the reverse of the reaction—citric to malic—may not have been noted heretofore. The malic to citric transformation has been recorded (**13**) when tobacco was cultured in the dark and also when tobacco was supplied with potassium malate (**11**).

The fact that major interconversions are here found to occur between citric and malic (evidently through the unidentified fraction) without material alteration of the total organic acid is evidence of a reversible equilibrium. The data of VICKERY *et al* (**25**) on the malic-to-citric acid reaction correspond fairly well with the Martius and Knoop hypothesis. This reaction proceeds to oxalacetic acid, to pyruvic acid, to a condensation product, and finally to citric acid. The present writers are without an opinion as regards the reversibility of this chain. In the 24-hour experiment the gain in "unidentified" as well and in citric acids equalled nearly the loss in malic. Also in the reverse reaction the loss of "unidentified" acids, added to the losses of citric (possibly preceding the full loss in citric) made up for gains in malic. This relation suggests that the "unidentified" acids are deserving of more attention than they sometimes receive. And, also, that

the key to the intermediate acids between malic and citric may be found in the "unidentified" group.

Ammonium versus nitrate nitrogen on base and organic acid accumulation

The organic acids have for many years been regarded as the great neutralizers or buffers of the Plant Kingdom. Recent papers by Ulrich (**20**) and by Pierce and Appleman (**10**) have dealt with phases of the inorganic cation-anion and organic acid relation. In other papers Clark (**4**) and Pucher *et al* (**12**), Vickery *et al* (**23**) and Wadleigh and Shive (**26**) have interested themselves in the influence of ammonium versus nitrate nitrogen on the organic acids. It has seemed to the writers, as well as to others undoubtedly, that the foregoing subjects might very well be closely related: *i.e.*, that the differences found in the quantity of organic acid might be in response to differences in total base and anion accumulation rather than to specific effects of the ammonium and nitrate ions. With this viewpoint the major inorganic cations and anions were included in the leaf analyses of the experiment reported in this section, table V.

A group of cotton plants (Stoneville 2B doubled haploids) was planted and thinned one to a pot in the greenhouse in tall four-gallon sand-filled stone jars and grown for 35 days on half-strength Hoagland's solution. Iron was supplied as magnetite mixed with sand. The pots were then taken out-doors (May 8) and divided into five groups of five plants each. One of these groups was cropped at this time for leaf analyses and weights. The other four groups were grown for an additional 20 days during which time they were differentially supplied with single salts plus boron and manganese. The four treatments were 8 m.e. per liter of the following: (1) $(NH_4)_2SO_4$, (2) NH_4Cl, (3) $Ca(NO_3)_2$, and (4) $NaNO_3$. Each of these solutions was supplied in sufficient excess to produce about 50 per cent. of drainage.

Although the plants made about three-quarters of their total growth during the last 20 days while supplied with single salts, table V, there was at the end of 20 days no evidence of any decline or deficiency. The ammonium sulphate plants were somewhat larger than those of the other treatments and they were especially deep green.

The salts carried by the stems and roots, together with those supplied by the tap water, contributed substantially to the maintenance of the initial mineral concentrations in the leaves. However, there were losses; among these is potassium but it remained sufficiently high in the leaves of all treatments for plant needs.

Between the two ammonium salts—and also between the two nitrate salts—there was little difference in the accumulations of total cations or of total inorganic anions. Likewise, the excess of cations over anions, and the concentrations and kinds of organic acids were similar within pairs. Possible exceptions are the extra amounts of "unidentified" organic acid in the Cl

TABLE V

INFLUENCE OF AMMONIUM AND NITRATE NITROGEN ON THE ORGANIC ACIDS OF COTTON LEAVES. MILLIEQUIVALENTS PER 100 GRAMS DRY WEIGHT*

TREATMENT	DRY WT. OF LEAVES	CATIONS					ANIONS					EXCESS OF CATIONS OVER INORGANIC ANIONS	ORGANIC ACIDS					FREE ORGANIC ACID
		CA	MG	K	NA	TOTAL	CL	NO_3	PO_4	SO_4	TOTAL		CITRIC	MALIC	OXALIC	UNIDENTIFIED	TOTAL	
END INITIAL 35 DAYS																		
Hoagland's Solution	19.3	198	45.6	77.2	7.8	329	11.3	23.6	24.5	71.9	131	198	63.1	56.6	12.7	119.6	252	54
END FINAL 20 DAYS																		
$(NH_4)_2SO_4$	98.5	174	34.0	29.7	10.4	248	11.4	9.5	23.9	86.5	131	117	40.5	79.1	15.1	58.3	193	76
NH_4Cl	78.5	174	33.6	26.2	8.7	243	54.9	12.2	26.2	25.2	119	124	42.2	73.3	16.0	83.5	215	91
$Ca(NO_3)_2$	82.5	197	37.4	36.9	9.3	281	23.3	12.3	25.6	22.9	84	197	55.3	96.0	17.6	88.1	257	60
$NaNO_3$	78.5	189	40.5	40.0	15.1	285	23.6	14.3	27.3	23.5	89	196	61.7	112.8	17.8	64.7	257	61

* All solutions were made up with tap water containing in m. e. per liter: Ca 0.2, Mg 0.1, Na 2.2, Cl 0.7, and SO_4 0.2. For the initial solution salts were added to give in addition: Ca 5.0, Mg 2.0, K 3.0, NO_3 7.5, PO_4 1.5, and SO_4 2.0.

and Ca leaves—but this is not especially important here. The main point is that neither SO_4 versus Cl nor Ca versus Na had any very great effects on the accumulation of the constituents mentioned.

Turning, on the other hand, to comparisons between the ammonium leaves and the nitrate leaves, it is found, in round numbers, that the ammonium leaves had nearly 40 m.e. (per 100 gms. of dry tissue) less of total bases and nearly 40 m.e. more of inorganic acids than did the nitrate leaves. These marked differences give rise to a 75 m.e. greater disparity between the cations and inorganic anions in the nitrate leaves than in the ammonium leaves. The question now arises—was this greater disparity in the nitrate leaves made up by an extra accumulation of organic acids? The next to the last and last columns ("Total" and "Free Organic Acids") of table V show that the disparity was compensated and with organic acids to spare.

The differences between the ammonium leaves and nitrate leaves in what is here designated as the "free organic acids"—*i.e.* the organic acids found over and above those required to balance the excess of determined inorganic bases over the determined inorganic acids—are not very large; the four values are respectively 76 and 91 m.e. for the two ammonium salts and 60 and 61 m.e. for the two nitrate salts. Cotton (**5**), in contrast with some other plants (**17**), accumulates very little ammonium ion; otherwise an explanation might be afforded for the greater "free organic acid" in the plants on the ammonium salts.

Returning to one of the original motives for the experiment, there was the question—does the substitution of ammonium nitrogen for nitrate nitrogen constitute a direct and immediate cause for a profound change in the organic acid regimen of the plant? Those who think so can point to the loss of 50 m.e., or 20 per cent., in the total organic acid when the plants were supplied with ammonium salts. On the other hand, those, like ourselves, who favor the neutralizing or buffering interpretation of organic acids will maintain that with a lessened difference between cations over inorganic anions from any cause, in this instance the substitution of ammonium, less organic acid is needed to maintain the characteristic acidity of the species and less will be found. By this latter interpretation ammonium versus nitrate would be regarded only as one of perhaps a number of factors that can set in motion a chain of events that leads to a similar result. Occasional plants like the cantaloupe (**10**), on the other hand, add to the difficulty of fully accepting any explanation for the behavior of organic acids as being entirely general.

The cotton plant is shown by this experiment to be capable of increasing greatly its store of organic acids to bring about a balance in the sums of bases and acids and thereby maintain its characteristic excess of organic acid over and above the inorganic cation-anion difference.

Respiration and translocation

If, beyond their role as neutralizing agents, the organic acids have a nutritional significance, either as intermediates in the formation of other

compounds or in respiratory activity, it might be expected that these acids would undergo substantial change during periods of prolonged darkness. Only a minor change in total acid is accredited (**13**) to excised tobacco leaves during prolonged darkness, but there was a notable shift from malic to citric. In excised rhubarb leaves (**14**) light was without effect; these leaves lost as much malic and other acids in light as in the dark. Whether grown in light or dark, plants from Narcissus bulbs (**24**) differed little in their organic acids after 28 days. On the other hand—whatever the physiological significance—marked diurnal variations have been found to occur in the organic acids of the succulent plants, the amount of organic acid being increased with reduced temperatures and with increased CO_2 of the atmosphere (**3**).

Little is known about the translocation of the organic acids largely because of difficulty of concluding in most instances whether the acids are actually moved about in the plant or disappear in one tissue while being coincidentally synthesized elsewhere. The results of PLATNITSKY (**11**) are direct in showing an accumulation of citric acid in excised tobacco leaves when the petioles were placed in a solution of potassium malate. In this instance the translocation to the leaves was presumably via dead tracheal elements rather than via the phloem. One of the major interests in the subject must center in the question of phloem transport and whether these acids are subject to polar movement analogous to the transport of the sugars.

VICKERY, *et al.* (**24**) suggest that tracer isotopes would be helpful in this problem but it is not clear how the technique could be applied to differentiate between tracheal and phloem movements. The experiment undertaken by the writers, table VI, endeavors to throw light on this subject by comparing during a period of darkness the losses of organic acids from detached cotton leaves with the simultaneous losses from attached leaves. The losses from the detached leaves are assigned to respiration and it is presumed that the additional loss from attached leaves can be accredited to translocation. The behavior of the organic acids as regards respiration and translocation is compared in these respects with that of carbohydrates.

The analyses, table VI, are of three sets of leaves, (1) fresh leaves gathered at 2 P.M., (2) excised leaves from the same plants after standing in the dark for five days with their petioles in distilled water, and (3) leaves which remained attached to the plants during the same five-day period in the same room. The temperature varied between 28° and 30° C.

Twelve Stoneville 2B plants were grown, one to a pot, in a manured soil in four-gallon jars until May 8 in the greenhouse. By this time the plants had started to flower and they were taken outdoors until used on May 20. All floral buds were removed during the May 8–20 period to increase the store of carbohydrates and thereby equip the plants and their leaves to withstand better the prolonged period of darkness.

As an aid to the collection of samples that would be uniform as regards the number of leaves of various size, age, and position all but the youngest

TABLE VI

EFFECT OF RESPIRATION (DETACHED LEAVES) AND RESPIRATION PLUS TRANSLOCATION (ATTACHED LEAVES) ON CARBOHYDRATES AND ORGANIC ACIDS DURING FIVE DAYS IN DARK. ALL VALUES RELATIVE TO DRY WEIGHT

		CARBOHYDRATES (GRAMS)						ORGANIC ACIDS (MILLIEQUIVALENTS)				
	DRY WEIGHT	HEXOSES	SUCROSE	STARCH	SUM	HEMICELLULOSE	CELLULOSE	CITRIC	MALIC	OXALIC	UNIDENTIFIED	TOTAL
INITIAL:												
Per 100 grams		1.04	1.04	25.62	27.70	9.40	4.74	82.0	57.6	10.2	37.3	187.1
Per 10 leaves	12.45	.13	.13	3.19	3.45	1.17	.59	10.2	7.17	1.27	4.65	23.29
RELATIVE, PER LEAF:												
Initial	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5 days detached	.79	2.23	1.30	.39	.47	.77	1.03	.59	.66	1.02	1.89	.89
5 days attached	.71	.31	.31	.07	.08	.83	1.03	.69	.57	.97	1.27	.78

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leaves were systematically marked, starting with the lowest healthy main-stalk leaf, with a white, yellow, or green tag taken in rotation from one plant to the next. The leaves of each tag color on six of the plants provided A samples and those on the other six plants the replicate B samples. The A and B plants during the 12-day period were in parallel rows between a pair of greenhouses. As one measure of sample uniformity, the dry weights of the A and B leaves were respectively 1.20 and 1.29 grams per leaf in the original collection, .96 and 1.00 grams per leaf after having been detached with their petioles in distilled water for five days in the dark, and .87 and .90 grams after having remained attached for five days in the dark.

Three inches of petiole were left on each leaf as a part of all samples, the petioles of the leaves to be detached for five days in the dark having been cut back to this length under water. The distilled water in the beakers was changed daily and the pH compared with that of distilled water which had stood alongside. There was no evidence of a change in pH to indicate movement of organic acid from the petioles to the water.

As shown by table VI, the detached leaves lost 21 per cent. of their original dry matter during five days in the dark. The attached leaves lost this amount and an additional 8 per cent. was translocated, *i.e.*, the three relative weights were 1.00, .79, and .71, respectively. Per ten attached leaves there was a loss of 3.62 grams in dry weight.

The sum of the sugars and starch was reduced to 47 per cent. of the original weight by respiration alone (the detached leaves) and to 8 per cent. of the original weight by translocation plus respiration (the attached leaves). Of the 3.62 grams total loss in dry weight of attached leaves, 3.17 grams are thus accounted for by the loss of sugars and starch.

There was a substantial loss in hemicellulose by respiration during the five-day period but there is no indication of any translocation within this group of constituents.

The changes in cellulose, if any, were well within the limits of error.

The total organic acids contrast sharply with starch and sugar in losses by both respiration and translocation. Whereas 92 per cent. of the sum of sugars and starch of the attached leaves disappeared during five days in the dark there was during the same period a disappearance of only 22 per cent. of the total organic acid from the same leaves. From the detached leaves only half as great a loss in organic acids occurred indicating that respiration and translocation were each responsible for the disappearance of 11 per cent. of the initial organic acid. Proportionately these losses were about the same in the A and B samples, as follows:

Group	Original	Detached	Attached
A	1.00	.91	.83
B	1.00	.86	.75

Irrespective of the various other physiological activities that may have accompanied respiration and translocation during the dark periods the data

suffice to show that relative to labile carbohydrates, the organic acids are nearly inert in both respiration and translocation. The changes in sums of starch and sugar as regards both respiration and translocation were in the order of five times as great as those of the organic acids.

It may be noted in table VI that oxalic was the only acid whose amount per leaf remained unchanged during the five-day period. This suggests that this acid in cotton, as in so many other plants, is largely in the form of inactive calcium oxalate. SCOTT *et al.* (**10**) have made the noteworthy observation that in orange leaves, prior to leaf drop, accumulated starch is replaced by accumulations of ensheathed calcium oxalate crystals.

Another opportunity for evaluating respiratory activity in cotton leaves is provided by table IV. These Acala and Stoneville leaves were gathered in mid-afternoon in wet and dry plots in the field in California and aliquots placed between wet and dry towels for 24 hours. Although in 24 hours these leaves lost an average of 21 per cent. of the sum of sugars and starch, there was no change whatever in the total organic acids. It would seem accordingly that the organic acids in cotton leaves may not be respired until the labile carbohydrates have largely disappeared or until some derangement of the vital activities of the protoplast has occurred.

CARBOHYDRATE-NITROGEN-ORGANIC ACID RELATIONS

Both carbohydrate and nitrogen data are reported in conjunction with the organic acids in tables II, III, and IV, and carbohydrate data in table VI. This has been done with the object of providing an opportunity to search for variations in any of these constituents which might be related to changes in the organic acids. The fact that we have failed to find consistent or important correlations between these data may serve to emphasize other relations, such as differences between inorganic acids and bases or shifted pH levels, as being more directly responsible for variations in total organic acid.

It is usually assumed that the carbohydrates are the precursors of the organic acids and some investigators believe that the organic acids are in turn the precursors of the amino acids. Variations in the concentration of one of these might or might not be reflected by variation in another. The formation of organic acids from carbohydrates, or of amino acids from organic acids, can presumably proceed only through the agency of enzymes. The conditions that increase or decrease enzymatic activity or determine the direction of an equilibrium reaction are often poorly defined and now customarily thought of as being related to the formation and activity of the hormones. The fact that transformations in plant constituents can occur one at the expense of another, and that several such changes can occur simultaneously, often from currently synthesized products, adds to the complexity of differentiating cause and effect. Having found that the organic acids tend to be relatively inert as regards respiration and translocation, the changes from or to other classes of compounds are probably too minor or too gradual for clear recognition of the sequence of events.

Conclusions and summary

Organic acids, on the basis of dry weight, have been found to be present in the cotton plant in successively lower concentration in the leaves, petioles, flowers, stems, roots, young bolls, seed kernels, and lint. Leaves of cotton frequently contain 20 per cent. of organic acid on the basis of dry weight.

Malic acid is usually present in greatest amount and tends to constitute 30 to 40 per cent. of the total organic acid.

Without materially changing the concentration of total organic acid, drought was found to increase malic and decrease citric. This transformation of critic to malic is found to be a reversible reaction and apparently proceeds through some intermediate acid or acids which are included in the unidentified group.

Leaves of cotton plants cultured on nitrate nitrogen had a greater sum of Ca, Mg, K, and Na, but a lesser sum of NO_3, Cl, SO_4, and PO_4 than did plants on ammonium nitrogen. This great increase in bases relative to inorganic anions was found to be compensated by an extra accumulation of organic acid. The result provides for the conclusion that a major role of the organic acids in cotton is as equalizing agents for differences in the sums of the inorganic acids and the bases. An excess of organic acid was found over and above that required to compensate for the insufficient inorganic acids. This excess undoubtedly contributed to the maintenance of the 5.00–5.60 pH characteristic of cotton leaves.

Other than for prolonged respiration, a change in the inorganic cation-anion balance was the only condition that was found in cotton to bring about a material change in the concentration of total organic acid.

Comparisons of the disappearance of the carbohydrates and organic acids from excised and attached leaves during five days in the dark point to the conclusion that the respiration and translocation of organic acids proceeds only about one-fifth as rapidly as does that of sugars. The carbohydrates were largely exhausted during the five days. It may be that respiration of organic acids does not occur until the labile carbohydrates are nearly exhausted. As evidence of this it was found in another experiment that there was no loss of organic acid in an initial 24-hour period during which a large store of labile carbohydrates was maintained.

Consistent correlations were not found between variations in the concentrations of the organic acids and the sugars, starch, or soluble and insoluble nitrogen.

Bureau of Plant Industry, Soils and Agricultural Engineering
and the Texas Agricultural Experiment Station
College Station, Texas

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THE TOPOGRAPHICAL TETRAZOLIUM METHOD FOR DETERMINING THE GERMINATING CAPACITY OF SEEDS

GEORGE LAKON

(WITH TWO FIGURES)

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In 1942 the author published the so-called tetrazolium method for determination of the germinability of seeds (**3**). This method is based upon the reduction in germinability of the seeds due to a gradual dying-off of the embryo (**2**). The topographical spread of necrosis of embryo tissues can be demonstrated by means of 2,3,5-triphenyl-tetrazolium-chloride. This compound is colorless but is reduced (hydrated) into the stable but nondiffusible red formazan by living cells (**3**). Coloration of a cell by tetrazolium is a definite indication of its viability because necrotic cells remain uncolored. By means of extensive experiments conducted over a period of years, the procedure has been perfected to demonstrate which parts of the embryo are at least sufficiently viable to make germination possible (**4**).

The recent publication of R. H. PORTER, MARY DURRELL, and H. J. ROMM (**1**) concerning the topographical tetrazolium method does not, however, present the method correctly in relation to several essential points. In order to correct these errors, the present author submits a short description of his method (**3**).

Use of the tetrazolium method merely involves preparation of the embryo of various species of seeds in such a way that all those parts which are decisive in the estimation of germinability be made visible. In most cereals the anterior face of the embryo must be visible. Since the pericarp is impermeable and opaque in most cereals, the embryo must be removed from the kernel with a lance-like scalpel or dissecting needle. Except in maize, a longitudinal section through the center of the embryo is not adequate because the lateral root primordia remain invisible. Only in oats is the pericarp sufficiently transparent to obviate excision of the embryo. The embryo of maize, however, possesses a simple root primordium, and the lateral root primordia (secondary radicles) in the mesocotyl are uniformly distributed. In this instance a longitudinal section provides the best preparation (**5**). The presoaking of the seeds in water is necessary only to facilitate the removal of the embryo. Initiation of germination is neither necessary nor significant for the test because even non-after-ripened cereals can reduce tetrazolium According to the method described below, the cross-sectioned grains of oats are placed in tetrazolium solution without pre-soaking. Only in the case of maize is the staining of the scutellum important in relation to germinability. This response of

the scutellum in other species of cereals is of no significance. The tetrazolium procedure, in contrast to the germination tests, practically eliminates experimental errors. Data from two tests of 100 kernels each treated in this manner have been found to provide the same accuracy as direct germination experiments carried out as four tests of 100 grains each. The reason for these results is that the topographic tetrazolium method practically eliminates systematic errors because the tests are independent of personal procedure; in the usual germination tests this individual error amounts to more than half of the total error according to RODEWALD (7). Only the sampling error remains and this will maximally be 50 per cent. higher with 200 kernels than with 400 kernels and therefore will not reach the amount of the total error of germination tests. In fact, statistical processing of several thousand tests has shown that in tetrazolium tests with 200 kernels each, the errors always were lower than in germination tests with 400 kernels.

For small cereals and maize the following procedure is most advantageously employed. A 1 per cent. aqueous solution of 2,3,5-triphenyltetrazolium-chloride is used. This reagent is light-sensitive. In handling it, exposure both to direct sunlight and prolonged exposure to diffused sunlight must be avoided. The solution when preserved in the dark remains unchanged for several months. It should have a pH value between six and seven in order to be properly reactive. According to the writer's extended investigations, a 1 per cent. solution at this pH-value proved most favorable, because it produced best reaction in all cases. For wheat, rye, and barley the desired number of grains is soaked in water from six to 18 hours which is done most conveniently during the night. The embryo is then excised by means of a sharp-bladed dissecting needle as follows: the needle is thrust through the pericarp and testa below the embryo; the scutellum is severed and the embryo removed. The embryo (except the scutellum) must remain undamaged and free from starchy endosperm and pericarp fragments. Embryos extracted in this manner are placed in tetrazolium solution. It is advisable to use small porcelain blocks having a round cavity about 30 mm. in diameter. Special care must be exercised to insure the continuous submersion of embryos in the solution. Prolonged contact with the air must absolutely be avoided.

The reaction reaches completion after about seven to eight hours at room temperature in the dark. The response can then be accurately determined. Only those embryos are germinable in which the plumule and adjacent tissue-bearing root primordia are stained. Coloration of a small portion of these tissues constitutes a positive test. The critical regions are indicated by hatched areas in figure 1. In wheat (fig. 1a) and oats (fig. 1d) (whose embryos possess an epiblast) the borderline between stem and root structures parallels the epiblast. In rye (fig. 1b) this line lies at the base of the upper root primordia; in barley (fig. 1c) this line is recognizable as a distinct constriction between shoot and root structures.

An inexperienced operator may at first consider the excision of embryos both difficult and tedious. The procedure is in reality extremely simple and can be learned in a very short time, often within a few hours. An experienced operator can prepare 200 embryos in about half an hour. Embryos which are decayed and soft cannot be extracted. These are classed as nongerminable.

Oats have a thin and transparent seed coat and hence excision of embryos is not necessary. The grain is evenly cut across by means of small nippers or scissors whereupon the two halves of the grain drop out of the hull. The dehulled halves of the grain which carry the embryo may then be placed in the tetrazolium solution without pre-soaking. It is advisable to place them in small glass dishes about 40 mm. in diameter. After some practice, the determination can be made in about 24 hours at room temperature. In some cases, however, it is advisable to extend the observation to 48 hours because with oats some individual kernels may show a faint coloring, which may cause doubt about the reaction. Criteria of germinability in these cases are the same as for other species of grains, namely coloration of shoot and root primordia without reference to the scutellum. The hatched portion of figure 1d indicates that part of the oat embryo which must at least be stained if the grain is to be classed as germinable.

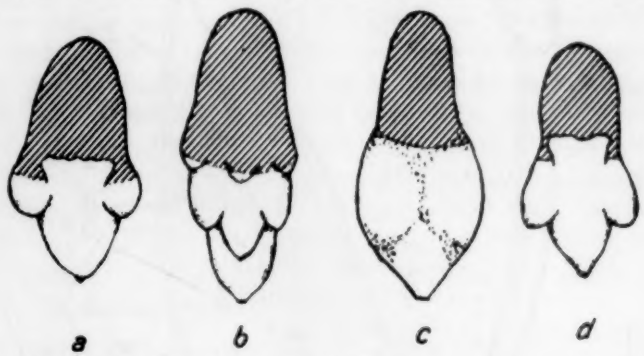

FIG. 1. Excised embryos of cereals, about ×13. a. Wheat; b. Rye; c. Barley; d. Oats. Those embryo parts which at least must show coloration after treatment with tetrazolium in order for the kernel to be germinable are shown by hatch marks.

In the case of maize (5) the grains to be tested are soaked in water for several hours, preferably over night. Then by means of a sharp scalpel, the kernels are cut longitudinally so that the embryo is bisected medianally throughout its entire length. One half of each grain is put into a 9 cm. petri dish and covered with tetrazolium solution until the grains are barely submerged therein. Contact of the tissue surfaces with the air must be avoided under all circumstances. The determination will be possible after three to four hours. Those grains are germinable in which the embryo is stained either completely or at least in the region of the shoot, including initials of secondary radicles and the scutellum. Coloration of

the corn scutellum, in contrast to other cereals, is significant and at least half of its area (the medial zone) should show coloration (fig. 2). The tissue connecting the germ and scutellum should be stained in its entirety. The minimum area requisite as a test of germinability is shown as the hatched portion of figure 2.

In especially urgent cases involving need for haste one may utilize tepid water for pre-soaking, and seed-parts submersed in the tetrazolium solution can be placed in an incubator at 30° C. In this manner the test may be shortened to a single day.

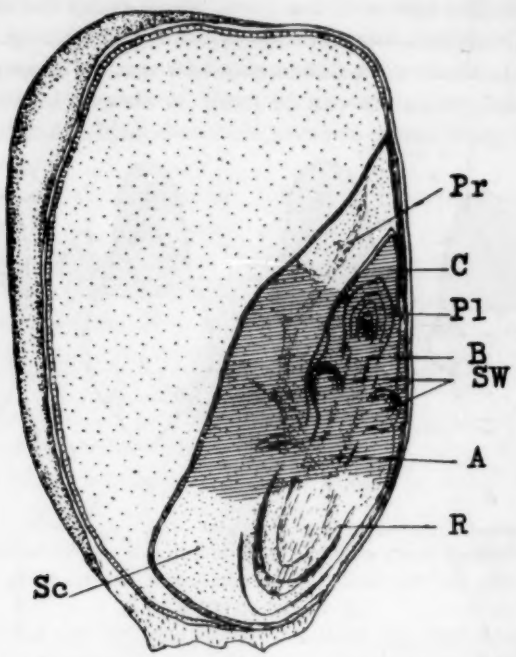

FIG. 2. Longitudinal median section through a kernel of corn. Sc, scutellum; Pr, procambial strand; C, coleoptile; Pl, plumule; B, base of plumule; A, transition from plumule to radicle; R, radicle; SW, initials of secondary radicles. That portion of the embryo which at least must show coloration after treatment with tetrazolium in order for the kernel to be germinable is shaded by hatch marks. About ×8.

The staining must be completed within 24 hours at the most. During longer periods, microorganisms may appear and obscure the reaction. This difficulty may, however, be overcome by transferring the seeds or embryos into a 1/1000 solution of mercuric chloride. In this case the test may be read at a later time.

Bacteria which multiply rapidly, especially in oat and maize tests even within the normal reaction time in tetrazolium, may cloud the picture in case of severe infection of the medium. Living bacteria, which are also stained by tetrazolium, may erroneously simulate staining of the cereal embryos or of the cut surface of the maize kernels. In such cases the

tetrazolium solution itself is stained red and this abnormal discoloration of the reagent enjoins caution. In such instances seed parts appear improperly stained, differing distinctly from normal coloration. Maize endosperm, for instance may be covered with minute red spots. This colored deposit can, however, be removed by gentle abrasion of the surface with a dissecting needle or scalpel and the correct test of underlying tissue can then be accurately determined.

In comparison with direct germination tests, the above tetrazolium procedure has the following advantages:

1. No great amount of space or large and complex apparatus is required. Tests can be conducted in a limited space with simple instruments such as porcelain blocks, petri dishes and small glass dishes, dissecting needles, scalpels, scissors or nippers. The tests themselves are simple and conclusive.

2. Execution is rapid even for large scale tests, small cereals requiring no more than 48 hours, and maize no more than one day at the most. In especially urgent cases the time may be considerably reduced by presoaking in tepid water and incubation at 30° C as described.

3. The method provides reliable and exact results. Even in the case of impediments to germination as in freshly harvested non-after-ripened cereals which are not immediately germinable but which possess inherent germinability of the grain (a condition which gave rise to the term "germinating potency" or "Keimpotenz" (**6**) in 1918), the eventual germination can be determined with certainty.

DEPARTMENT OF SEED RESEARCH AND SEED TESTING STATION
AGRICULTURAL COLLEGE
STUTTGART-HOHENHEIM, GERMANY

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POTASSIUM IN CITRUS TREES

A. R. C. Haas

(WITH FOUR FIGURES)

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Introduction

Potassium is included in the fertilizer program of many citrus orchards without any very definite proof that a real need for K exists. The symptoms that characterize other deficiencies such as zinc, manganese, and iron in citrus are more or less definite and specific. The present investigation is largely concerned in the production and identification of K-deficiency symptoms when citrus trees are grown in sand cultures, in the hope that such a knowledge of leaf patterns may permit a reliable diagnosis of K-deficiency symptoms in citrus orchards.

It is desirable to understand more fully the chemical relationships of K to other constituents in citrus trees. This investigation has continued without interruption since 1920, use being made of various artificial cultures and of trees in the field. As a result of these studies, more attention is being given to the scion and rootstock relation, to the flowers as affected by the rootstocks and to the state of the nutrient balance. The importance of nitrogen fertilization on the K and Mg content in citrus leaves, flowers and fruit is herein referred to. This paper includes a study of lemon trees that heretofore have received little or no attention as regards their K needs. The bearing of K and other elements on the important problem of fruit size is also discussed.

Literature

A review of the literature discloses that a deficiency of K in orange or grapefruit trees in various types of cultures is accompanied by a large array of symptoms. Among these are: a checked or retarded root growth, gum exudations on twigs and trunk, splitting of twigs, twig die-back, temporary iron chlorosis in young leaves, resinous spotting in leaves, tendency of chlorophyll to fade and of leaves to take on a bronze-yellow color, leaf-scorch, lowering of resistance of leaves to wind injury, excessive loss of the oldest leaves principally near the time of flowering, and the production of small leaves (**12, 13, 14,** and **22**); tucking of the leaf blade and the twisting or crinkling of leaves (**4**), rust-like appearance of leaves (**3**), increased losses of fruit (**13**), fewer, smaller and earlier maturing fruit (**2, 3, 9, 21,** and **24**), and the occurrence of resinous areas on the exterior of the peel (**22**).

From the standpoint of composition a deficiency of K in orange or grapefruit trees was accompanied by a number of chemical changes. Somewhat increased amounts of Ca (**22**), Mg, Na, and N (but not of P) accumu-

lated in citrus leaves (**6**). The expressed juice of K-deficient leaves (**22**) was more acid than that of leaves containing abundant K. A deficiency of K in the nutrient medium was accompanied by a decreased K content in the juice of orange fruit (**24**).

In normal healthy orange leaves (**12**) the K content decreases as the maturity of the leaves increases. The roots and rootlets are the last to be depleted of K when the deficiency of K becomes acute (**22**). Large concentrations of K normally occur in the outer portion of the peel of orange fruit and the percentage of K in the fruit (**14**) was increased by K fertilization of the soil.

A deficiency of K occurred (**22**) when K was present but not in sufficient concentration relative to that of another element such as calcium or sodium. Application to the soil of calcium and magnesium (**11**) suppressed uptake of K in grapefruit leaves.

In the absence of an adequate supply of minor elements (**11**), K was absorbed in excess of its needs. Large accumulations of K frequently accompany a deficiency of Mg (**16**) in citrus leaves. K accumulation decreases materially when Mg is increased. Thus the presence of large amounts of K in the leaf is frequently an unreliable index that excessive K in the soil is the sole cause of the K accumulation. In reality the absorption of some other element such as Mg may be insufficient (**16**) and the more so when N is also deficient (which reduces the Mg absorption) and as a consequence K tends to accumulate.

Through the use of artificial cultures, critical values (**9**) for K in citrus leaves were set up: 0.26 to 0.35 per cent. K in the dry matter was designated as indicating a very slight K deficiency in healthy-appearing trees; 0.52 to 0.86 per cent. was taken as an indication of no deficiency of K. The range 0.35 to 0.52 per cent. was given no consideration. The K range (**10**) of 2.15 to 3.63 per cent. was designated as being excessive K.

No certain evidence of K deficiency has as yet been observed in citrus orchard trees in California (**8**). By means of a survey (**7**) of hundreds of citrus orchards, the leaves of most trees were found to contain 0.4 to 1.0 per cent. in their dry matter whereas only two cases showed K low enough as to suggest a K deficiency on soil types not in common use for citrus orchards.

The K content in the dry matter of the leaves and flowers of citrus trees having the same scion variety is dependent to a large degree on the nature of the rootstock variety (**15**, **17**, and **18**). The K percentages in the dry matter of mature Valencia orange leaves ranged from 0.565 to 0.958 depending upon the rootstock variety; in lemon leaves the range was 0.306 to 1.073 per cent., whereas in grapefruit leaves it was 0.410 to 1.024 per cent. The K content in dry matter of Eureka lemon flowers ranged from 1.321 to 1.940 per cent. and in Valencia orange flowers 1.181 to 1.836 per cent. depending on the nature of the rootstock variety. Thus mature lemon leaves from healthy trees on certain rootstocks contained 0.306 per

cent. K and would be slightly K deficient if the values 0.26 to 0.35 per cent. are accepted as critical values whereas similar leaves of trees on other rootstocks a few rows distant contained 1.073 per cent. K and would be considered as having ample K.

Experimental methods

POTASSIUM DEFICIENCY.—A group of seven galvanized iron containers 20 inches in diameter by 26 inches deep (coated on the interior with asphalt and on the exterior with aluminum) were used for the outdoor sand cultures in each of which three rooted Valencia orange cuttings were planted on April 8, 1941 (table I). Eight outdoor cultures similar to these were set up for Eureka lemon and three rooted cuttings were planted in each culture. The various K levels were continued from the time the cuttings were planted until the termination of the experiment on June 12, 1946. Smaller trees were obtained than might otherwise have been the case, but the experiment was thus freed from any effects of a large backlog of K previously accumulated within the trees.

Every sand culture had bottom drainage and received large volumes (nine to 18 liters) of culture solution every few days. The culture solution was essentially that used by REED and HAAS (**23**) (KNO_3, $MgSO_4 \cdot 7H_2O$, NaCl; $Ca(NO_3)_2 \cdot 4H_2O$; KH_2PO_4; plus minor elements) with the modification that the KNO_3 and KH_2PO_4 were omitted, that the entire NO_3 content of the solution was supplied as calcium nitrate and that 10.5 p.p.m. instead of 105 p.p.m. PO_4 was supplied as the primary calcium salt. One-tenth p.p.m. each of boron, manganese, and copper, 5.5 p.p.m. iron, and 0.25 p.p.m. zinc were used as the minor elements and the pH of the culture solution added did not exceed 4.5. Potassium sulphate was used to give various concentrations of K in the culture solution.

In these cultures containing Valencia orange or Eureka lemon cuttings the concentrations of K were: 0.1, 1.0, 3.0, 6.6, 10.0, 15.0, and 50.0 p.p.m. In the cultures containing Eureka lemon cuttings a 2 p.p.m. culture was also included. At various times collections were made of flowers, leaves, fruit and other samples that were cleaned, dried, ground and analyzed as in previous studies (15–19). The green leaves collected were fully mature, thick, and leathery in texture, capable of producing new growth and were picked just prior to the production of a new cycle of growth. The scorched yellow leaves were mature leaves that were about to abscise.

Six outdoor solution cultures were also set up in which Valencia orange trees were grown on sour orange rootstocks at the following K levels (from K_2SO_4): 6.25, 12.50, 18.75, 25.0, 31.5, and 37.5 p.p.m. K respectively. The culture solution used was similar to that of the other previously mentioned cultures. These solution cultures were set up purposely for flower collections.

LARGE POTASSIUM CONCENTRATIONS.—This study was initiated on account of the report (**9**) that injury results when large concentrations of K are

used in cultures. An experiment with five outdoor sand cultures was set up on August 29, 1946. In each culture was planted a Valencia orange tree on sour orange rootstock. The culture solution contained 54 p.p.m. Mg from the sulphate, 10.5 p.p.m. PO_4 from primary calcium phosphate, 600 p.p.m. NO_3 from calcium nitrate, and 25 p.p.m. N from $(NH_4)_2SO_4$. Potassium sulphate solution was added so as to give K concentrations ranging

TABLE I

EFFECT OF LOW CONCENTRATIONS OF POTASSIUM IN THE CULTURE SOLUTION ON THE COMPOSITION OF THE LEAVES AND ROOTLETS OF VALENCIA ORANGE CUTTINGS GROWN IN LARGE OUTDOOR SAND CULTURES (EXPERIMENT 1)

K IN CULTURE SOLUTION	IN DRY MATTER							
	K	Ca	Mg	TOTAL P	K	Ca	Mg	TOTAL P
	Mature green leaves, September 25, 1945				Mature yellow, scorched leaves, April 30, 1946			
p.p.m.	%	%	%	*p.p.m.*	%	%	%	*p.p.m.*
0.1	.....	.....	.....	.....	0.186	.....	.....	.....
1.0	0.217	3.866	0.473	1530	0.253	4.840	0.519	.....
3.0	0.293	3.763	0.453	1570	0.163	5.068	0.497	.....
6.6	0.268	5.032	0.482	1250	0.091	6.174	0.547	.....
10.0	0.239	5.881	0.576	850	0.135	6.450	0.616	.....
15.0	0.303	5.210	0.525	890	0.105	7.252	0.693	.....
50.0	0.323	5.949	0.516	680	0.230†	.....	.....	.....
	Mature green leaves, June 13, 1946				Mature yellow, scorched leaves, June 13, 1946			
3.0	0.353	.....	.....	.....	0.149	.....	.....	1170*
6.6	0.389	3.882	0.391	.....	0.119	5.515	0.552	1020
10.0	0.319	4.138	0.487	.....	0.173	.....	0.575	730
15.0	0.380	4.635	0.473	.....	.....	.....	.....	.....
50.0	0.444	4.644	0.501	.....	.....	.....	.....	.....
	Mature yellow, scorched leaves, April 15, 1946				Small rootlets, June 17, 1946			
0.1	.....	.....	.....	.....	0.244	0.489	0.584	1730
1.0	.....	.....	.....	.....	0.186	0.505	0.562	1300
3.0	.....	.....	.....	.....	0.177	0.469	0.522	1130
6.6	0.106	6.688	0.583	.....	0.158	0.459	0.567	960
10.0	0.074	6.952	0.657	.....	0.181	0.479	0.779	860
15.0	0.094	7.568	0.704	.....	0.225	0.457	0.623	750
50.0	.....	.....	.....	.....	1.248	0.507	0.541	820

* Total P values for green leaves from cultures with 3 to 50 p.p.m. K inclusive were: 1500, 1420, 1150, 930, and 750 p.p.m. respectively.
† Very few affected leaves.

from 150 to 350 p.p.m. inclusive. A few fruit were available for analysis and were picked on May 27, 1948.

On June 3, 1948 the trunks of the trees were cut back to about 14 inches above the bud union. A K level ranging from 150 to 450 p.p.m. inclusive was established on March 5, 1948 when the culture solution was altered so as to contain: 108 p.p.m. Mg from the nitrate (550 p.p.m. NO_3), 636 p.p.m. Ca from the nitrate (1972 p.p.m. NO_3), 52.5 p.p.m. PO_4 from primary

calcium phosphate, 0.1 p.p.m. each of Mn, B, and Cu, 5 p.p.m. of Fe and 0.25 p.p.m. of Zn. The growth response after nine months has thus far been excellent.

Experimental results

Potassium deficiency

Valencia orange cultures.—In June 1946 when the Valencia orange cuttings in sand culture were harvested, the approximate height of the tops increased from 21 to 38 inches for the low to high level of K supplied respectively. Table I (experiment 1) shows that the total P values for green or yellow leaves decreased whereas Ca and Mg increased as the K level in the culture solution increased. Green leaves generally contained higher percentages of K than yellow leaves. The highest K value for green leaves occurred with the highest concentration of K in the culture solution. Yellow leaves were found in all of the cultures, however, in decreasing numbers as the K concentration in the culture solution increased. Only at the highest K concentration was there a marked increase in the K content in the rootlets. The K values in the leaves were less than were generally found in leaves in the field. High percentages of Ca and Mg may, as shown in previous studies, accompany low K but not necessarily so, as is shown in table I.

Distortion, curling, puckering, chlorophyll fading, yellowing, tipburn, rusty-brown resinous spotting, dark gum deposits on leaves, tucking, scorching, and abnormal leaf fall were the leaf reactions chiefly present (fig. 1) at the extremely low K level (0.1 p.p.m.) and decreasing rapidly at slightly higher K levels. It is possible that the leaf distortions such as twisting, curling, etc. may be the accompaniment of excessive carbohydrate accumulation as occurs when boron is deficient or as in rooted citrus leaves in which the absence of buds prevents new top growth. Twig weakness and die-back occurred where leaf symptoms were extreme. Arnot (1) has described a twig die-back in Australia that was associated with basal necrosis in weak orange shoots.

The number of Valencia orange flowers decreased markedly as the K level in the culture solution was decreased. In 1944 the flowers opened first in the culture with the highest K level and progressively later in the cultures maintained at the lower K levels. Cultures at the three lowest K levels (0.1, 1.0, and 3.0 p.p.m. K) were very slow to produce new growth and flowers. In 1943 all of the orange flowers were picked whereas in 1944 the flowers were counted but not picked. From March 16 to April 9, 1943 at the increasing K levels shown in table I the number of flowers produced in the various cultures were: 1, 15, 147, 152, 511, 724, and 166 respectively whereas from April 7 to May 30, 1944 the number of flowers produced per culture were: 4, 3, 52, 933, 1289, 2328, and 2473 respectively. In general as the supply of K given to the trees increased, they produced a very rapidly increasing number of flowers. This may be due to the larger

size of the high K level plants and along with this an increased carbohydrate production and an increased K content, for it will be pointed out that at higher K levels the flowers contain increased amounts of dry matter and increased percentages of K.

As the K levels increased, the percentages of K in the dry matter of the Valencia orange flowers increased, for at the 3, 15, and 50 p.p.m. K levels,

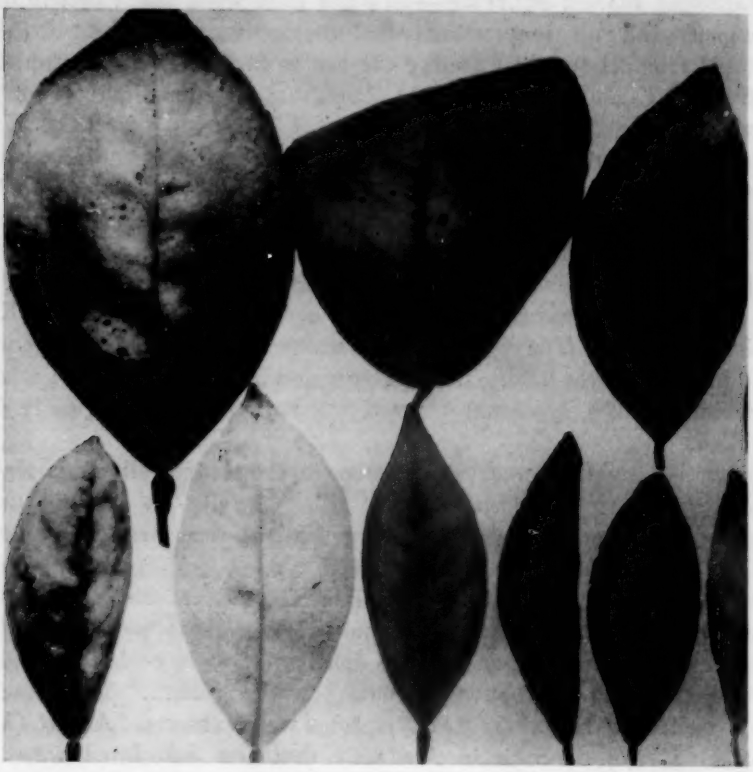

FIG. 1. Effect of low concentrations of K on leaves collected April 27, 1946 from rooted Valencia orange cuttings grown in large outdoor sand cultures. In upper row: left, chlorophyll fading, yellow and resinous spotting; middle leaf, curling and resinous spotting; right, yellowing and tip burn; lower row: left, tucking; second from left, yellowing; second from right, gum along edges and midrib, and elsewhere. Leaves showing these severe symptoms had a K content usually near or below 0.25 per cent. whereas the K content of healthy mature leaves generally exceeded this. The extremely high mobility of K in leaves and the K changes that accompany maturity render the setting up of rigid narrow critical values of doubtful significance.

the dry matter of the flowers contained 0.821, 1.191, and 1.951 p.p.m. K respectively. In a second sampling of flowers at the 6.6, 10.0, 15.0 and 50.0 p.p.m. K levels, the dry matter of the flowers contained 0.958, 0.971, 1.055, and 1.713 per cent. K respectively.

As the K levels increased, the percentages not only of K but also of dry matter in the Valencia orange flowers increased, for at the 15 and 50 p.p.m.

K levels, the fresh weight of the flowers contained 16.90 and 18.62 per cent. of dry matter respectively. In another flower sampling at the 6.6, 10.0, 15.0 and 50.0 p.p.m. K levels, the fresh weight of the flowers contained 17.65, 18.75, 20.00, and 21.77 per cent. of dry matter respectively.

The K level at which the Valencia orange trees were grown appeared to affect the Mg content in the dry matter of the flowers. In the first sampling of flowers, at the 15 and 50 p.p.m. K levels, the Mg content in the dry matter was 0.183 and 0.253 per cent. respectively whereas in the second sampling, at the 6.6, 10.0, 15.0 and 50.0 p.p.m. K levels, the Mg content in the dry matter was .146, .186, .161, and .238 per cent. respectively. Thus

TABLE II

EFFECT OF VARIOUS LOW CONCENTRATIONS OF POTASSIUM AS THE SULPHATE IN THE CULTURE SOLUTION ON THE FRUIT OF VALENCIA ORANGE CUTTINGS GROWN IN LARGE OUTDOOR SAND CULTURES (EXPERIMENT 1)

K IN CULTURE SOLUTION	No. OF FRUIT USED	AVERAGE FRESH WEIGHT PER FRUIT	IN DRY MATTER OF PEEL				IN DRY MATTER OF PULP			
			K	Ca	Mg	TOTAL P	K	Ca	Mg	TOTAL P
Fruit collected June 7, 1945										
p.p.m.		*gm.*	%	%	%	*p.p.m.*	%	%	%	*p.p.m.*
6.6	9	100.1	0.225	0.696	0.072	1050	0.735	0.400	0.105	2090
10.0	12	97.2	0.236	0.750	0.076	960	0.695	0.394	0.100	1810
15.0	20	86.8	0.260	0.681	0.093	730	0.858	0.353	0.093	1430
50.0	16 small	87.4	0.737	0.577	0.106	670	1.291	0.244	0.091	1000
	16 large	118.7	0.691	0.544	0.129	600	1.310	0.283	0.088	1120
Fruit collected June 11, 1946*										
3.0	1	67.9	0.281	.....	.....	1720	0.557	.....	.....	2520
6.6	15	80.8	0.299	0.755	0.083	1180	0.637	0.358	0.094	2020
10.0	20	83.6	0.257	0.784	0.099	920	0.781	0.386	0.094	1900
15.0	20	80.1	0.293	0.796	0.108	750	0.851	0.374	0.082	1320
50.0	16 small	76.8	0.927	0.503	0.121	650	1.399	0.239	0.097	1130
	16 large	116.9	0.869	0.444	0.106	580	1.511	0.209	0.104	1230

* Average diameters of fruit middles on October 27, 1945 were for the cultures at the 6.6, 10.0, 15.0 and 50.0 p.p.m. K: 4.125, 4.176, 4.495, and 4,923 cm. respectively.

at increasing K levels the Mg percentages tend to increase both in the dry matter of the flowers and leaves.

Further evidence that increasing K levels are accompanied by increased K content in the dry matter of Valencia orange flowers was obtained from outdoor solution cultures in which sour orange was the rootstock. In the first sampling, at the 12.50, 18.75, 25.00, 31.50, and 37.50 p.p.m. K levels the percentages of K in the dry matter were found to be: 1.815, 2.493, 2.936, 3.043, and 3.238 per cent. respectively and in a sampling two weeks later at the 6.25, 12.50, and 31.50 p.p.m. K levels, the K percentages found were 1.231, 1.543, and 2.450 respectively.

Some of the Valencia orange fruits grown in the K-deficient sand cultures had resinous spots in the surface of the rind. As shown in the foot-

note in table II, the diameters at the fruit middles increased as the K level increased. This may be a consequence of the decreasing loss of leaves as the K levels increased.

In 1945 the percentages of peel decreased as the K level increased whereas in 1946 this tendency was not so pronounced. This tendency toward thinner peel is seen in the following results. Valencia orange fruit picked on June 7, 1945 from the sand cultures at the 6.6, 10.0, 15.0, and 50.0 p.p.m. K levels had the following fresh weights of peel as percentages of the fresh weight of the whole fruit: 34.91, 33.36, 30.76, 26.56 (small fruit

FIG. 2. The effects noted on June 8, 1945 of various concentrations of K (1.0, 2.0, 3.0, 6.6, 10.0, 15.0, and 50.0 p.p.m. respectively) as the sulphate on the growth of rooted Eureka lemon cuttings grown outdoors in large sand cultures (experiment 2, table III). At 0.1 p.p.m. K the appearance was slightly worse than at 1.0 p.p.m. and the culture was omitted in the figure in order to reduce the length of the photograph. Leaf distortion occurred only in the lower end of the range of K concentration, where also considerable necrosis of new shoots, twig splitting and die-back were evident.

from the 50 p.p.m. culture) and 29.28 (large fruit from the 50 p.p.m. culture) respectively; whereas on June 11, 1946 the percentages for the cultures at the 3.0, 6.6, 10.0, 15.0 and 50.0 p.p.m. K levels were: 25.04 (1 fruit), 30.89, 27.24, 24.07, 26.69 (small fruit from the 50 p.p.m. culture), and 27.93 (large fruit from the 50 p.p.m. culture) respectively.

In the collection of fruit for analysis, it was found that the 50 p.p.m. culture had quite a number of small as well as large fruit and therefore samples of the two groups were kept separate (table II). Up to and inclusive of the 15 p.p.m. K level, any increase in the percentage of K in the peel was very small or absent whereas in the pulp an increased percentage

of K was associated with increased K levels. Total P in the peel and pulp decreased as the K level increased. The percentages of K, Ca, and the total P content in the peel of the small fruit from the 50 p.p.m. culture were slightly greater than those in the peel of the larger fruit from the same culture. However, in the pulp the percentages of K and the total P content are slightly greater in the larger fruit.

LEMON CULTURES.—The approximate heights on May 4, 1945 of the tops of the Eureka lemon cuttings in sand cultures maintained at 0.1 to 50.0

FIG. 3. The effect (as seen on April 5, 1943) of low concentrations of K on rooted Eureka lemon cuttings grown in large outdoor sand cultures. Mature leaves were yellowish to brownish green and curled. New shoots were weak and curved, and with necrotic areas or splits along the yellow twigs, frequently resulting in the collapse of new shoots. Flowering shoots were spindly or absent.

p.p.m. K levels increased from 20 to 72.5 inches for the low to high level of K supplied, respectively. Figure 2 shows the relative growth and appearance of these lemon cuttings. At very low K levels there was a curling and yellowing of the leaves and a dying of young shoots (fig. 3). The flowering shoots were long and spindly and bore few small or rudimentary leaves. The new shoots were weak and often curved, the twigs being yellow as if sunburned, and with necrosis or splits near the base. Figure 4 shows a curved new shoot such as were also found on K-deficient trees in the field.

Table III shows the K content in the leaves of the lemon cuttings at the various K levels in the culture solution. The calcium content in the leaves was relatively low. There was a tendency for the percentages of magnesium to increase as the K level increased.

The total P content in the dry matter of various portions of lemon cuttings and fruit are given in table IV. In agreement with the results in tables I and II for Valencia orange cuttings and fruit, the total P content in the dry matter of lemon cuttings and fruit shows a decrease as the K

FIG. 4. Weak, curved new shoots, often with yellow (as if sunburned) areas showing splits, were found May 3, 1943 on rooted lemon cuttings grown in large outdoor sand cultures that received a solution low in K. Such yellowish, curved, and sometimes split shoots have been found in orchard trees, the leaves of which are low in K. These trees were not deficient in boron as the culture solution contained 0.1 p.p.m. and the low K levels frequently were accompanied by slight boron toxicity symptoms in the leaves. Leaf analysis showed adequate boron.

level was increased. The average middle diameters and the number of lemon fruits on October 27, 1945 for the cultures at the 3.0, 6.6, 10.0, 15.0, and 50.0 p.p.m. K levels were: 2.23 cm. (1 fruit), 3.22 cm. (21 fruits), 3.80 cm. (39 fruits), and 4.11 cm. (38 fruits) respectively.

Eureka lemon fruits, when tree-ripe, were larger with increasing K concentrations in the culture solution as shown in table V. A marked in-

crease occurred in the percentage of K in the dry matter of lemon peel when the K level was increased from 15 to 50 p.p.m. A small increase in the percentage of Mg and a decrease in the total P content were found in the peel as the K level was increased. In the pulp the percentage of K increased considerably as the K level was increased.

POTASSIUM IN LEAVES OF CITRUS ORCHARDS.—The described experiments

TABLE III

EFFECT OF POTASSIUM ON THE COMPOSITION OF EUREKA LEMON CUTTINGS GROWN IN LARGE OUTDOOR SAND CULTURES (EXPERIMENT 2)

K IN CULTURE SOLUTION	IN DRY MATTER					
	K	Ca	Mg	K	Ca	Mg
	%	%	%	%	%	%
	Mature leaves, Sept. 26, 1945			Bark (trunk, large branches), Jan. 22, 1946		
0.1	.....	.....	.....	.142	2.602	.158
1.0	.269	3.621	.423	.161	2.476	.151
2.0	.239	4.177	.519	.193	2.403	.200
3.0	.198	4.187	.492	.180	2.359	.210
6.6	.298	3.977	.524	.180	2.088	.256
10.0	.308	4.195	.566	.190	2.236	.268
15.0	.314	4.365	.528	.230	2.321	.321
50.0	.603	3.675	.619	.750	2.864	.460
	Mature leaves, Jan. 22, 1946			Bark of large roots, Jan. 22, 1946		
0.1	.220	3.716	.397	.....	2.602	.158
1.0	.254	3.957	.421	.159	2.671	.155
2.0	.271	3.953	.478	.212	2.247	.168
3.0	.269	3.915	.505	.196	2.040	.179
6.6	.295	4.365	.597	.210	2.003	.207
10.0	.274	4.568	.617	.....	1.878	.214
15.0	.360	4.262	.575	.257	1.965	.232
50.0	.743	3.357	.572	.578	1.886	.303
	Small twigs and branches, Jan. 22, 1946			Rootlets, Jan. 22, 1946		
0.1	.106	1.500	.275	.142	1.040	.....
1.0	.144	1.383	.247	.163	0.883	.325
2.0	.124	1.523	.250	.245	0.673	.382
3.0	.132	1.252	.253	.249	0.613	.454
6.6	.137	1.291	.255	.260	0.676	.580
10.0	.151	1.492	.250	.216	0.690	.475
15.0	.493	1.894	.361	.234	0.734	.387
50.0	.173	1.799	.301	.806	0.789	.524

with sand and solution cultures have added to our previous knowledge regarding the symptoms and K composition under low potassium levels. Use has been made of these results in attacking the problem of the K status in orchard trees.

The K-deficiency symptoms in a citrus tree in the field, unless very severe, do not remain static: they change according to the occurrence of

TABLE IV

TOTAL PHOSPHORUS CONTENT IN THE DRY MATTER OF EUREKA LEMON CUTTINGS GROWN IN LARGE OUTDOOR SAND CULTURES THAT RECEIVED CULTURE SOLUTIONS CONTAINING VARIOUS AMOUNTS OF POTASSIUM AS THE SULPHATE (EXPERIMENT 2)

K IN CULTURE SOLUTION	MATURE LEAVES COLLECTED SEPT. 26, 1945	MATURE LEAVES COLLECTED JAN. 16, 1946	GREEN TWIGS COLLECTED JAN. 16, 1946	BARK OF TRUNK AND BRANCHES COLLECTED JAN. 16, 1946	ROOTLETS COLLECTED FEB. 7, 1946	ROOT BARK COLLECTED FEB. 7, 1946	SILVER TO TREE-RIPE FRUIT (1945–1946)	
							PEEL	PULP
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
0.1	2090	2080	3580	1263	1200	900	.....	.....
1.0	2180	2220	3400	1263	1325	875	.....	.....
2.0	2060	2130	2850	1213	1713	888	.....	.....
3.0	1680	2010	2650	1088	1525	850	1694	4000
6.6	1470	1470	1400	938	1313	850	1063	A±2520
10.0	1240	1230	800	813	988	775	A 813	B 2770
15.0*	1210	1350	650	825	963	800	B 788	A 2075
50.0	920	1170	760	700	1038	725	A 680	B 2063
							B 650	C 2160
							.....	A 2000
								B 1950
								.....

* A third fruit sample consisted of very small green whole fruit: 1360 p.p.m. P in dry matter.
± A, B, C, are different fruit samples.

TABLE V

EFFECT OF VARIOUS LOW CONCENTRATIONS OF POTASSIUM IN THE CULTURE SOLUTION ON THE FRUIT OF EUREKA LEMON CUTTINGS GROWN IN LARGE OUTDOOR SAND CULTURES (EXPERIMENT 2)

K IN CULTURE SOLUTION	NO. OF FRUIT USED	AVERAGE FRESH WEIGHT PER FRUIT	IN DRY MATTER OF PEEL				IN DRY MATTER OF PULP			
			K	Ca	Mg	TOTAL P	K	Ca	Mg	TOTAL P
TREE-RIPE FRUIT COLLECTED MAY 2, 1945										
p.p.m.		*gm.*	%	%	%	*p.p.m.*	%	%	%	*p.p.m.*
6.6	4	56.5	0.388	0.895	0.132	1230	0.560	.....	.....	.....
10.0	13	73.7	0.382	1.027	0.144	970	0.830	0.334	0.117	.....
15.0	13	97.7	0.395	1.047	0.158	770	1.140	0.348	0.133	1900
50.0	10	137.4	0.830	0.826	0.164	620	2.041	0.260	0.116	.....
TREE-RIPE FRUIT COLLECTED NOVEMBER 29, 1945										
15.0	9	62.0	0.420	1.202	0.168	.....	1.038	0.355	0.099	.....
50.0	20	90.0	1.085	0.866	0.169	.....	1.626	0.270	0.117	.....
TREE-RIPE FRUIT COLLECTED JANUARY 15, 1946										
6.6	20	34.1	0.276	0.792	0.116	.....	0.684	0.314	0.104	.....
10.0	32	52.9	0.262	0.898	0.139	.....	0.774	0.313	0.104	.....
GREEN TO SILVER STAGE FRUIT COLLECTED JANUARY 15, 1946										
10.0	7	48.7	0.212	0.957	0.158	.....	0.723	0.355	0.106	.....
GREEN* (ABOUT SAME SIZE AS TREE-RIPE) FRUIT COLLECTED NOVEMBER 29, 1945										
15.0	16	51.3	0.294	1.131	0.163	.....	1.032	0.339	0.102	.....

* Very small green fruit collected November 29, 1945 from the culture at the 15 p.p.m. K level showed (for 9 fruit averaging 30.8 gm. each): 0.688% K, 0.768% Ca, and 0.149% Mg in the dry matter of the whole fruit.

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the several growth cycles, the stage of leaf maturity, and the K stress at or near the period of flower production. Mature affected leaves abscise prematurely and are replaced by leaves in which the symptoms may not be evident (unless the K deficiency is very acute) until the leaves approach maturity.

TABLE VI

POTASSIUM, CALCIUM, AND MAGNESIUM CONTENT OF HEALTHY-APPEARING AND OF AFFECTED CITRUS LEAVES COLLECTED FROM THE SAME TREES

SAMPLE NO.	SCION VARIETY	LEAF CONDITION	PLACE OF COLLECTION	IN DRY MATTER			K + Ca + Mg**
				K	Ca	Mg	K
				%	%	%	%
1	Lemon	Healthy	Santa Barbara	0.375	4.828	0.605	15.5
2	Lemon	Affected	Santa Barbara	0.242	5.111	0.708	25.0
3	Lemon	Healthy*	Carpinteria	0.285	4.978	0.232	19.3
4	Lemon	Affected*	Carpinteria	0.209	5.203	0.273	27.2
5	Lemon	Healthy	Goleta	0.255	5.834	0.459	25.7
6	Lemon	Affected	Goleta	0.187	6.446	0.521	38.3
7	Lemon	Healthy*	E. of Santa Paula, W. of Sespe	0.482	4.510	0.242	10.9
8	Lemon	Affected*	E. of Santa Paula, W. of Sespe	0.338	5.304	0.281	17.5
9	Eureka lemon	Healthy	Santa Paula	0.312	5.473	0.339	19.6
10	Eureka lemon	Affected	Santa Paula	0.182	5.922	0.355	35.5
11	Lemon	Healthy	W. of Sespe	0.334	6.011	0.344	20.0
12	Lemon	Affected	W. of Sespe	0.219	6.352	0.380	31.7
13	Lemon	Affected	W. of Sespe	0.232	5.903	0.322	27.8
14	Lisbon lemon	Affected	Santa Paula	0.191	6.447	0.346	36.6
15	Nucellar Frost Eureka lemon	Healthy	Springville, S.E. of Saticoy	0.297	8.180	0.500	30.2
16	Nucellar Frost Eureka lemon	Affected†	Springville, S.E. of Saticoy	0.227	8.256	0.509	39.6
17	Valencia orange	Healthy*	Santa Paula	0.537	6.406	0.056	13.0
18	Valencia orange	Affected	Santa Paula	0.289	7.247	0.448	27.6
19	Lemon	Affected†	Escondido	0.138	5.554	0.456	44.6
20	Lemon	Affected†	Escondido	0.146	4.910	0.449	37.7
21	Lemon	Affected†	N. Carlsbad	0.271	5.004	0.389	20.9
22	Lemon	Affected†	Escondido	0.156	5.339	0.508	38.9
23	Lemon	Healthy	Santa Barbara	0.447	4.927	0.479	13.1
24	Lemon	Affected†	Santa Barbara	0.281	6.631	0.485	26.3
25	Marsh grapefruit	Healthy	Oroville	0.390	4.806	0.587	14.8
26	Marsh grapefruit	Affected	Oroville	0.272	6.133	0.773	26.4
27	Lemon	Affected	Porterville	0.247	.....	.....	.....
28	Lemon	Affected	Escondido	0.248	5.137	0.436	23.5
29	Lemon	Affected	Escondido	0.255	5.075	0.451	22.7
30	Eureka lemon	Affected	Santa Paula	0.247	6.020	0.309	26.6
31	Lemon	Affected	Upland	0.273	.....	.....	.....

** Credit for suggesting this column is given Mr. D. J. Raden of Grower's Advisory Service Laboratory of Swift and Company, Los Angeles, California.

* Magnesium-deficiency symptoms present.

† Very severely affected leaves.

The numerous symptoms produced in these and previous studies such as abnormal leaf fall, destruction of new growth, gum formation, etc. have formed a broad background of symptoms that have proved of value in the K diagnosis of orchard trees (19). For more than a quarter century, attempts have been notably unsuccessful in finding California citrus trees in which the leaves had a low K content and consequently the application of potassium fertilizers to soils of citrus orchards has lacked recommendation.

The appearance of some of the symptoms (artifically produced in cultures) in a certain lemon orchard suggested that the K content of the leaves might be low and upon analysis they were found to contain low K. These lemon leaves also had a gum-spotting that as yet has not been satisfactorily produced in artificial cultures, and its significance will require further study. Fortunately other orchard samples of citrus leaves from time to time submitted for diagnosis, have shown these same leaf symptoms. Now the leaves of these orchards have been found to possess a low K content. Instruction of various individuals regarding these leaf symptoms has resulted in the locating of a fairly large number of widely scattered affected orchards.

Analyses (table VI) of affected leaves have left no doubt that citrus leaves in certain areas may have a low K content and that such leaves are quite readily diagnosed. Other investigators have now taken up the problem from there and first shall try to increase the K content of affected trees in various ways and then to note whether such increased K content will improve the health of the trees.

LARGE CONCENTRATIONS OF POTASSIUM.—A few fruit were available from Valencia orange trees grown in sand cultures with high K levels: 150, 200, 250, and 350 p.p.m. of K respectively. The fresh weight per fruit at these various K levels were: 189, 133, 256, and 182 grams respectively. The potassium percentages in the dry matter of the peel at the increasing K levels were: 1.397, 1.569, 1.907, and 2.118 per cent. respectively. At the 200, and 300 p.p.m. K level, the percentages of Ca in the dry matter of the peel were: 0.465, and 0.389 per cent. respectively whereas the percentages of Mg were: 0.079, and 0.084 per cent. respectively.

The dry matter of the pulp of the above Valencia oranges contained: 1.799, 1.804, 2.169, and 2.342 per cent. of K respectively; 0.260, 0.242, 0.141, and 0.168 per cent. of Ca respectively; and 0.090, 0.077, 0.082, and 0.101 per cent. of Mg respectively.

These data show that the K percentages in the peel and pulp increased as the K level increased whereas the Ca percentages tended to be lower. The fruit were fully colored and free from coarse rough rind or diseases (5). The fruit were not unduly large and were of good quality. The high K levels (as used) were not accompanied by injurious effects on these fruit, when the matter of nutrient balance (25) was given consideration.

Other lemon and orange trees when grown in a similar manner in sand cultures with K levels extending over the range from 50 to 700 p.p.m. in-

elusive, have not fruited as yet but have made very satisfactory growth when the nutrient solution had the proper balance.

In large outdoor Hanford sandy loam soil cultures of Valencia orange on sour orange rootstock, a single fruit occurred in each of the cultures that received 150 and 300 p.p.m. K respectively. A lower total P content in the dry matter occurred at the higher K level: peel, 940 and 870 p.p.m.; pulp, 2030 and 1820 p.p.m. respectively. This trend is in harmony with the results given for sand cultures in table II. The K percentage in the dry matter of the flowers (2.936 and 3.331 respectively) increased as the K level increased which also is in agreement with results obtained with sand and solution cultures.

POTASSIUM IN LARGE AND SMALL FRUIT.—Since low K levels are accompanied by a reduction in fruit size, it is of interest to know whether the size of fruit (large and small) collected from the same tree bears any relation to their K content. About fifteen small ripe fruit of approximately the same middle diameters and the same number of large ripe fruit of a given large middle diameter were calipered and picked from the same tree in the orchard at Riverside, California. The fruit of lemon, Valencia orange, and Washington Navel orange trees were thus collected, and after being washed with distilled water and wiped dry, the peel was separated from the pulp. The fresh weights were obtained and the peel of all samples and the pulp of one sample were oven-dried at 65° C and then ground and prepared for analysis as previously described (**15–19**).

On April 17, 1946 the values for the peel as a per cent. of the fresh weight of the whole fruit was for tree ripe lemons (1 14/16 inch middle diameter) 39.23 per cent. and for larger fruit (2 11/16 inch middle diameter) 44.07 per cent. Small Washington Navel oranges (2 6/16 inch) and larger fruit (2 13/16 inch) had 33.39 and 35.57 per cent. of peel respectively whereas small Valencia oranges (2 6/16 inch) and larger fruit (2 13/16 inch) had 28.18 and 29.73 per cent. of peel respectively. On July 18, 1946, small Valencia orange fruit in comparison with larger fruit gave the values of 25.16 and 29.49 per cent. respectively. Thus, in every case, on a fresh weight basis, the larger fruit had the greater percentage of peel.

The dry matter of the peel of large fruit did not consistently show the larger percentages of K; for large vs. small fruit on April 17, 1946: 0.983 vs. 1.067 (lemons): 0.640 vs. 0.745 (navel oranges); 0.652 vs. 0.561 (Valencia oranges) and on July 18, 1946: 0.940 vs. 1.080 (Valencia oranges). Thus it appears that some factor other than K (such as, total P, N, or carbohydrates) will require investigation as regards their effects on fruit sizes.

In the dry matter of the peel the Ca percentages were for large vs. small fruit: 0.826 vs. 1.038 (lemons); 0.690 vs. 0.653 (navel oranges); 0.797 vs. 0.912 (Valencia oranges); and on July 18, 1946: 0.865 vs. 0.877 (Valencia oranges). The Mg percentages in the peel of large vs. small fruit were: 0.107 vs. 0.149 (lemons); 0.094 vs. 0.099 (navel oranges); 0.095 vs. 0.100 (Valencia oranges); and on July 18, 1946: 0.112 vs. 0.104 (Valencia oranges).

The total P content in the dry matter of the peel was always greater in the larger fruit, the values (p.p.m.) for large vs. small fruit being: 690 vs. 610 (lemons); 750 vs. 650 (navel oranges); 630 vs. 500 (Valencia oranges); and on July 18, 1946, 900 vs. 870. These results for the peel of fruits of healthy trees in the field are not in agreement with the results in table II for fruits grown with various K levels in sand cultures.

In the dry matter of the pulp in the large vs. small Valencia orange fruit of July 18, 1946, the K percentage values were respectively 1.721 vs. 1.611; the Ca percentage values 0.340 vs. 0.285; and the Mg percentage values 0.105 vs. 0.107; whereas the total P (p.p.m.) values were: 1830 vs. 1500. The results for the pulp are thus in fair agreement with those for the pulp of large and small fruits of the 50 p.p.m. K sand culture in table II. In other Valencia orange fruit collections in Orange County where much of the fruit was of small size, the dry matter of the pulp, from trees in an orchard bearing uniformly large fruit, contained the highest total P content (1715 p.p.m.) of any of the six samples collected. In any effort to relate fruit size to causal factors, a large number of possible agents will needs be taken into consideration as has been pointed out by Hodgson (**20**).

POTASSIUM IN ORCHARD TREES AS AFFECTED BY FERTILIZERS.—A study was made of the effects of fertilizer application to orchard soil on the content of K and other constituents in the tree. Collections were made of Washington Navel orange leaves (150 per sample) and flowers (240 per sample) and of Valencia orange fruits (15 per sample) from trees in the fertilizer trials at the Citrus Experiment Station at Riverside. The leaves had just reached maturity and were thick and leathery in texture. The twigs bearing these leaves were capable of producing new growth and the leaves were picked just prior to the production of a new cycle of growth. All samples were cleaned, dried, ground, and analyzed as in previous studies (**15–19**).

When nitrogenous fertilizers were added to the soils of citrus plots, the percentages of K in the dry matter of the mature leaves, the flowers, and the fruits were less than in those from trees on the non-fertilized plots (table VII). The leaves and flowers from trees in the fertilized plots were higher in calcium than those from trees in the non-fertilized plots.

The percentage of magnesium in the leaves was increased by nitrogen fertilization. This is most important for it explains in part the partial or the sometimes complete recovery from magnesium deficiency that may take place when nitrogen is supplied in adequate amounts to citrus orchards. In citrus flowers, however, the percentages of magnesium decreased when nitrogenous fertilizers were used.

The phosphorus content in the leaves was greatly reduced when nitrogenous fertilizers were applied to the soil. The dry matter of the fruit from the non-fertilized plot contained the highest percentages of K, Ca, and Mg and the highest total P content.

Summary

Solution, sand, and soil cultures, representing a range of K concentrations, were used in studying the effects of K on the growth and composition

TABLE VII

THE CONTENT OF POTASSIUM AND OTHER CONSTITUENTS IN WASHINGTON NAVEL ORANGE LEAVES AND FLOWERS AND IN WHOLE VALENCIA ORANGE FRUITS AS AFFECTED BY NITROGEN APPLICATIONS TO ORCHARD SOILS

ORCHARD PLOT	SOIL TREATMENT	IN DRY MATTER			
		K	CA	MG	TOTAL P
MATURE WASHINGTON NAVEL ORANGE LEAVES COLLECTED AT C.E.S., RIVERSIDE, CALIFORNIA ON OCTOBER 9, 1942					
		%	%	%	*p.p.m.*
D 12	No fertilizer and cover crop	1.406	4.385	0.239	1540
D 32	Calcium nitrate (3 lbs. N) and cover crop	0.820	5.321	0.298	1030
D 46	Ammonium sulfate (3 lbs. N) and cover crop	0.816	5.355	0.305	880
MATURE WASHINGTON NAVEL ORANGE LEAVES COLLECTED AT C.E.S., RIVERSIDE, CALIFORNIA ON JULY 2, 1946					
G 38	No fertilizer	2.353	3.292	0.206	3030
J 2	Sodium nitrate (3 lbs. N)	0.942	3.945	0.352	1280
H 30	Sodium nitrate (3 lbs. N), gypsum 1946	0.908	4.182	0.345	1070
J 40	Urea (3 lbs. N)	0.672	4.755	0.355	1010
J 32	Calcium nitrate (3 lbs. N)	0.601	5.073	0.364	980
WASHINGTON NAVEL ORANGE FLOWERS COLLECTED AT C.E.S., RIVERSIDE, CALIFORNIA ON APRIL 13, 1942					
D 12	No fertilizer and cover crop	2.269	0.358	0.204	.....
L 14	No treatment	2.361	0.433	0.221	.....
D 46	Ammonium sulfate (3 lbs. N) and cover crop	1.910	0.508	0.191	.....
H 42	Urea (3 lbs. N), potassium sulfate and cover crop	1.870	0.535	0.199	.....
D 32	Calcium nitrate (3 lbs. N) and cover crop	1.695	0.486	0.177	.....
K 14	Sodium nitrate (3 lbs. N) and cover crop	1.687	0.490	0.179	.....
D 42	Calcium nitrate (3 lbs. N) and cover crop	1.665	0.485	0.195	.....
H 46	Urea (3 lbs. N), phosphate and cover crop	1.630	0.573	0.187	.....
COMPOSITE OF PEEL AND PULP OF SEVEN VALENCIA ORANGE FRUITS COLLECTED FROM TREES IN FIELD 1, C.E.S., RIVERSIDE, CALIFORNIA, AUGUST 21, 1946					
J, R30, T1	No fertilizer, no cover crop	1.017	0.601	0.102	1870
J, R42, T1	Calcium nitrate (5 lbs. N), and cover crop	0.961	0.543	0.098	1360
I, R40, T1	Calcium nitrate (3 lbs. N) and cover crop	0.955	0.469	0.090	1370

of the leaves, flowers, and fruit of orange and lemon trees. The underlying hope in such an investigation was to artificially produce a sufficient array of symptoms that would be of assistance in diagnosing a deficiency or excess of K in orchard trees.

The symptoms produced by means of low K, offered the first clue or suggestion that leaves in some citrus orchards may be low in K content. In lemon leaves in the field a peculiar gum-spotting not as yet produced in artificial cultures, tended to mask the symptoms of K deficiency as they were known in artificial cultures. This gum spot alone is now of value in the detecting of leaves low in K content in citrus orchards, and now a considerable number of such affected orchards are known.

For over a quarter century it has heretofore been quite impossible to detect low K in citrus trees except by chemical analysis alone. Now a K deficiency can be diagnosed quite readily by means of symptoms whereas chemical analysis can be used to lend confirmation of the low K content. The present study has raised at least two important questions and other investigators have now taken up the problems: (1) as to how to increase the K content of affected trees, and (2) to learn whether such K increases can remedy the trouble.

A deficiency of K was found to be associated with small fruit sizes and poor set. The enormous leaf fall especially near the flowering period in such cultures and the effect of such leaf abscission on the organic and mineral reserves in the tree, may account in large measure for the reduction in fruit sizes and set.

With orange trees in sand cultures, the number of flowers produced, the dry matter as a percentage of the fresh weight of the flowers and the percentage of K in the dry matter of the flowers, decreased as the K level was greatly reduced in the deficiency range. As the K level was greatly decreased, the total phosphorus content in the leaves, peel, and pulp was greatly increased. When the K level was the same, (50 p.p.m. K in table II), the percentage of K and the total phosphorus content in the pulp of Valencia oranges, from cuttings grown in sand culture, were slightly greater in the larger size fruit.

As the K level was increased with the same nitrogen content in the culture solution, there was a tendency for a higher magnesium percentage in orange and lemon leaves.

When the K level was increased from 15 to 50 p.p.m. in sand cultures, a marked increase occurred in the percentage of K in the dry matter of lemon peel and pulp that was accompanied by a marked decrease in calcium content (table V).

An excess of K was not produced in artificial cultures with fairly large concentrations of K, provided the solution was properly balanced. High concentrations of K were, however, found in citrus leaves of orchard trees in other studies (**16**) in which a magnesium deficiency occurred and when the supply of minor elements was inadequate (**11**).

On a fresh weight basis, large fruit had a greater percentage of peel than small fruit when both lots of fruit were collected from the same tree in the orchard. The dry matter of the peel of large fruit did not consistently show the larger percentages of K whereas it always showed a greater total phosphorus content. The dry matter of the pulp of large fruit contained slightly more K and considerably more total phosphorus than that of the pulp of small fruit from the same tree in the orchard.

Lower percentages of K were found in mature leaves, in flowers, and in the fruit of trees in nitrogen-fertilized plots than in those of trees in non-nitrogen-fertilized plots. The leaves and flowers from trees of nitrogen-fertilized plots were the higher in calcium whereas in the leaves from trees in such plots the total phosphorus content was greatly reduced and the percentage of magnesium was increased. This increase in available magnesium upon adding nitrogen fertilizer to the soil of orchard trees may explain in part the partial or complete recovery from magnesium deficiency that may occur when nitrogen is adequately supplied.

When K levels of Valencia orange trees in sand cultures were: 150, 200, 250, and 350 p.p.m., the percentages of K in the peel at the various K levels were: 1.397, 1.569, 1.907, and 2.118 per cent. respectively whereas in the pulp the values were: 1.799, 1.804, 2.169, and 2.342 per cent. respectively. No physical abnormalities or diseases could be attributed to the high K levels when the nutrient balance was adequate.

In sand cultures, K levels that are being maintained over a range of 50 to 700 p.p.m. have thus far produced no injury when the nutrient levels of the culture solution are high and the solution is well balanced.

UNIVERSITY OF CALIFORNIA,
CITRUS EXPERIMENT STATION,
RIVERSIDE, CALIFORNIA.

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GROWTH AND CHEMICAL COMPOSITION OF *ANANAS COMOSUS* (L.) MERR., IN SOLUTION CULTURES WITH DIFFERENT IRON-MANGANESE RATIOS[1]

(WITH EIGHT FIGURES)

C. P. SIDERIS AND H. Y. YOUNG

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Introduction

Pineapple plants grown in highly manganiferous soils become chlorotic and produce fruits which are small and of an inferior quality (**29**). Pineapple chlorosis is prevented in commercial plantations by the application of sprays of an iron sulphate solution.

Chlorosis of pineapple plants may develop, also in non-manganiferous soils with pH values higher than 5.5, especially when treated with fertilizers with residual alkaline reactions; high pH causing oxidation of Fe^{++} to Fe^{+++} with subsequent precipitation of the latter. Hence, ammonium sulfate with residual acidic reaction is preferred to sodium nitrate with residual alkaline reaction as a pineapple plant fertilizer.

Manganese chlorosis may be caused, according to JOHNSON (**29**) and KELLEY (**30**), by conversion of the water soluble ferrous to the water insoluble ferric iron by exchanges of electrons with manganese; a reaction comparable with that at high pH.

The results below, concerned with the growth of pineapple plants at different iron-manganese ratios and the chemical composition of the tissues under the various conditions, suggest that manganese chlorosis may result from a replacement of iron by manganese in the pyrrole ring of protoporphyrin 9, a precursor of chlorophyll.

Review of literature

JOHNSON (**29**) explains manganese chlorosis in pineapples as the result of the oxidation of ferrous to ferric iron by manganese at pH values above 5.5 whereby pineapple plants deprived of available iron fail to produce chlorophyll. KELLEY (**30**), discussing pineapple chlorosis, states that "yellow pineapples from soils containing a high percentage of manganese yielded poorly, had lower amounts of starch and chlorophyll but the xanthophyll content was the same in both chlorotic and green leaves." GILE (**16**) was of the opinion that manganese chlorosis of pineapples in Hawaii was due, in part, to iron deficiency in the plant, induced by manganese, while the lime-induced chlorosis in Puerto Rico was the lack of iron in the plant due to calcium carbonate diminishing the availability of iron in the soil.

[1] Published with the approval of the Director as Technical Paper No. 185 of the Pineapple Research Institute, University of Hawaii.

McGeorge (**37**) concluded from Johnson's and Kelley's data that pineapple chlorosis on manganiferous soils was due to a great assimilation of lime and that the principal physiological disturbance was the greater immobility of iron in the plant caused by the excessive lime content of the leaves and stalk, since iron was present in equally available form in both manganiferous and non-manganiferous soils of equal pH.

Other plants than pineapples have been found susceptible to manganese chlorosis. Rippel (**45**) observed that oats made chlorotic by high Mn in solution cultures contained almost equal amounts of iron as green plants, and concluded that Mn had not interfered with Fe absorption. Haas' (**20**) results indicate that excessive amounts of Mn caused chlorosis in citrus although Fe was added in comparable amounts, and, also, that when Mn was deficient in the leaves less iron had accumulated. Somers, Gilbert and Shive (**60**) and Somers and Shive (**61**) attributed manganese chlorosis in soy beans to certain iron-manganese ratios which, depending on the prevelance in the concentration of either element, may cause iron or manganese toxicity. Lindner and Harley (**32**) are of the opinion that in pear, apple and spiraea leaves, chlorosis may be induced by high potassium levels by replacing the iron on the enzyme responsible for chlorophyll formation, thereby inactivating the enzyme. Twyman (**64**), after reviewing the literature on the iron-manganese balance and its relation to chlorosis, concluded that "further investigations are necessary on the effects of this balance on the growth of plants and the occurrence of pathological symptoms, so that the deficiency disease concerned may be better understood."

Bennett (**5**) showed in prune trees that iron-manganese ratios may vary considerably (1.6–0.1) more than those indicated by Somers and Shive (**61**) without development of severe chlorosis symptoms but with Mn concentrations higher than 100 γ which presumably was toxic, chlorosis could be induced promptly. Sideris (**59**) observed more retardation of Fe translocation from roots to leaves in cultures with, than without Mn.

Experimental methods

Cultural methods.—Crowns (vegetative organs produced at the apical end of fruits) from a clone of uniform weight were grown in two sets of nutrient solutions, one containing ammonium salts and the other nitrate salts as sources of nitrogen but all other elements supplied in equal amounts to both sets. The ammonium-N set contained 0.132 g. of $(NH_4)_2SO_4$ and 0.172 g. $CaSO_4 \cdot 2H_2O$, and the nitrate-N set 0.236 g. of $Ca(NO_3)2 \cdot 4H_2O$ per liter of solution. Other salts equally applied to both sets per liter of solution were 0.246 g. $MgSO_4 \cdot 7H_2O$, 0.174 g. K_2SO_4, 0.068 g. KH_2PO_4, 0.0028 g. $ZnSO_4 \cdot 7H_2O$, and 0.0038 $Na_2B_4O_7 \cdot 10H_2O$. Iron and manganese were added to the various cultures as indicated in figure 1.

The various nutrient solutions placed in 4-gallon porcelain crocks, one

plant per crock, were aerated continuously and changed at three-week intervals. At the end and beginning of such intervals the plants were weighed and samples of the solution cultures were collected for determinations of the amounts of iron and manganese removed by the plants.

ANALYTICAL METHODS.—Iron and manganese were determined colorimetrically, the former by the nitroso-R-salt (**52**) and the latter by the periodate method (**3**).

Iron and manganese in the tissues were fractionated into sap soluble and insoluble fractions; the former extractable by acetone but not the latter.

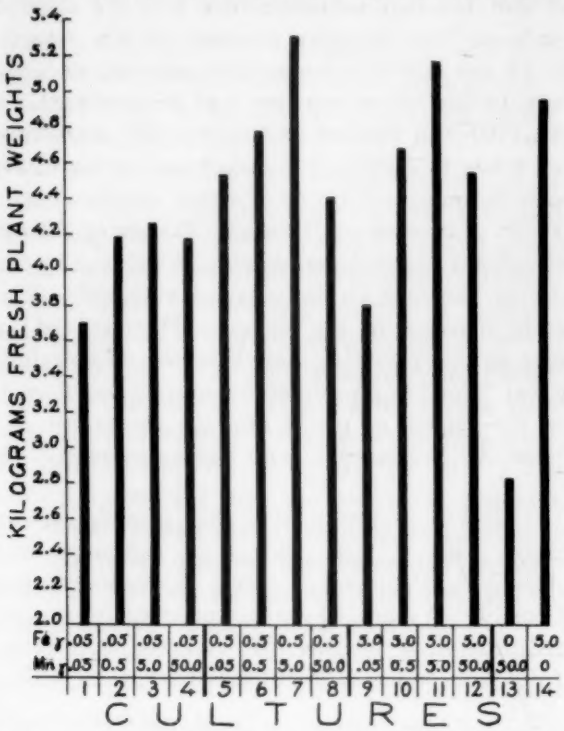


FIG. 1. Plant weights of cultures.

Also, protein bound iron or manganese fractions, the former reported previously (**58**), was obtained by extraction of the proteinaceous matter of the tissues (**57**) and determination of its iron and manganese content.

Sap soluble iron was obtained by mixing 10 grams of leaf tissues with 80 ml. of acetone in a Waring Blendor. The mixture after maceration for 15 minutes was filtered through No. 32 Whatman paper and the residual matter washed with more acetone until the removal of all green color was complete. The filtrate, after evaporation of the acetone in a platinum dish, was ashed and both iron and manganese determined by the methods above mentioned. Also, determination for total iron and manganese were made in different aliquots of the same leaf tissues after ashing in order to provide

means for the calculation of insoluble iron or manganese from the difference between total and acetone extractable (sap soluble) iron or manganese.

The acetone extraction method for soluble Fe and Mn was adopted after certain trials had showed that both elements were soluble in acetone in much greater amounts than those found in pineapple tissues.

Potassium, magnesium, and phosphorous were determined by methods reported previously (**50, 53, 55**), whereas calcium was determined by the official method (**3**).

Reducing sugars were determined by the method of QUISUMBING and THOMAS (**43**) and sucrose was calculated after treatment with invertase, from the difference between total and reducing sugars. For the estimation of starch the method of PUCHER and VICKERY (**42**) was employed. Ascorbic acid was determined with sodium 2,6-dichlorobenzenoneindophenol on a 10 ml. aliquot of the filtered extract obtained by mixing 10 grams of leaf tissues with 100 ml. of a 5 per cent. solution of oxalic acid and grinding in a Waring Blendor.

Chlorophyll was extracted and measured according to SCHERTZ's (**47**) method.

Results

PLANT GROWTH.—Plant weights, in figure 1, varied considerably in the different cultures presumably on account of different iron-manganese ratios. The data indicate that in cultures 1, 5, and 9 supplied with very low manganese (0.05 γ) and in cultures 4, 8, 12 and 13 with very high (50.0 γ) manganese plant weights were depressed presumably in the former cultures by Mn-deficiency and in the latter by Mn-toxicity. The slight depressions in plant weights of cultures 9 and 10 with high (5.0 γ) Fe might indicate toxicity or improper balance between iron and manganese or between iron and other mineral nutrients. However, culture 14, with high Fe and minus Mn produced very satisfactory plant weights, indicating that neither the presence of high Fe (5.0 γ) caused toxicity or the lack of Mn, except the small contaminations in the C.P. nutrient salts, Mn-deficiency.

With respect to any definite amounts of manganese requirements for pineapple plants the data are inconclusive, because in certain cultures plant weights increased as the amounts were raised from 0.05 to 5.0 γ except culture 14 which, although lacking Mn, had almost as good plant weights as cultures 7 and 11. These results presumably suggest that extremely small amounts of Mn suffice in the presence of adequate Fe concentrations to produce satisfactory growth. Iron-manganese ratios ranging from 1 to 1 or from 1 to 10 as in cultures 11 and 7, respectively, may yield almost equally good results as long as the concentration of manganese is not abnormally high.

Under Hawaiian field conditions water soluble iron concentrations in most soils are ordinarily lower than 0.05 γ and manganese, in manganiferous soils, as high or higher than 50 γ. Total iron may range, according to

KELLEY (**17**), in manganiferous soils, from 18 to 26 and manganese from 2.5 to 9.0 per cent. and in the non-manganiferous soils iron from 20 to 35 and manganese from 0.06 to 1.17 per cent.

Because moisture conditions, water soluble iron-manganese ratios, the active plant nutrient fractions, and concentrations of other ions (PO_4^{---}, CO_3^{--}) reacting with Fe or Mn vary considerably in different soils at different pH values results obtainable by the solution culture procedure

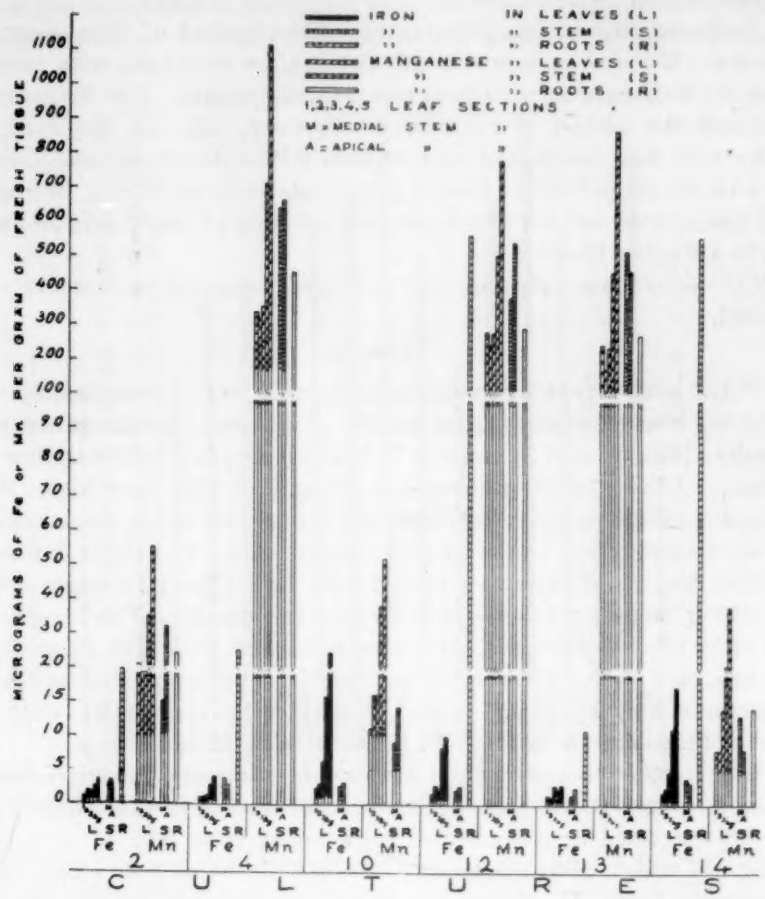


FIG. 2. Total Fe of Mn in leaf (L), stem (S), or root (R) tissues of cultures.

indicate approximations and not true conditions that plants encounter in soils.

IRON AND MANGANESE IN PLANTS.—The data in figure 2, depicting iron and manganese in different plant sections of the various cultures, reveal that Fe and Mn increased in the tissues with higher concentrations of these elements in the culture solutions. However, cultures 13 and 14, the former minus-Fe and the latter minus-Mn, contained small amounts of

these elements not supplied to the nutrient solutions, but which presumably occurred as contaminations in the C.P. nutrient salts.

The results indicate clearly that manganese did not accumulate in the roots but was translocated to the leaves, whereas iron was greatly immobilized in the roots as indicated in figure 2 and very small amounts moved to the leaves. The translocation of iron from roots to leaves, although very small in all cultures, was inhibited more in cultures 12 or 4 with high Mn than in cultures 10 or 2 with low Mn. In no case was iron translocation inhibited by manganese to a point of absence in the

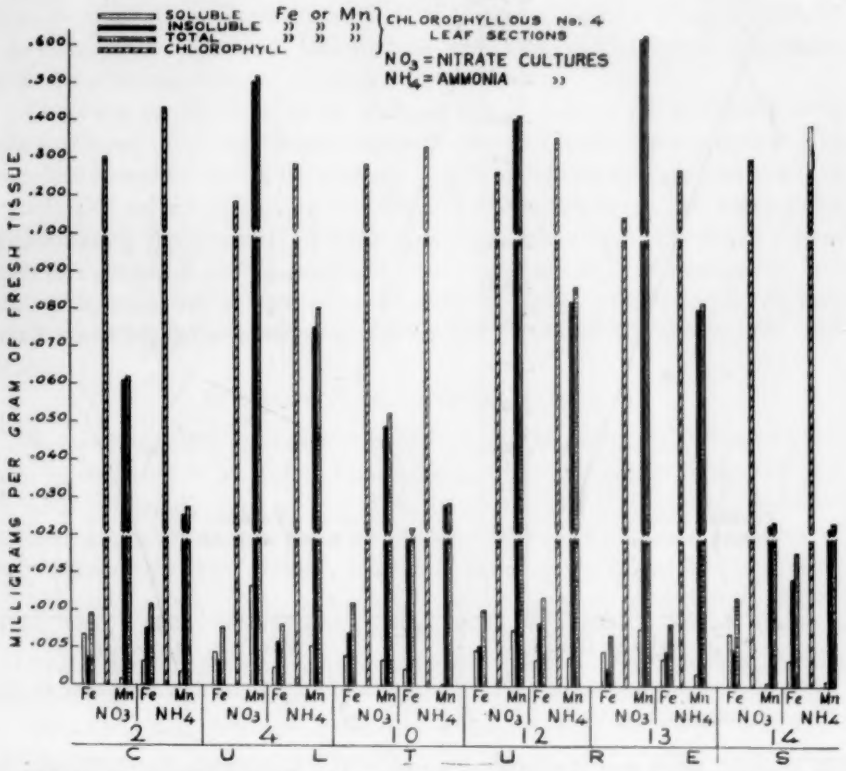


FIG. 3. Iron or manganese and chlorophyll in active (D) leaves.

leaves. However, the antagonistic effects of manganese, whatever the nature of their reactions in the plant cells, could be easily traced in the chlorophyllous regions of the leaves by the development of mosaic chlorotic patterns in the early stages which coalesced in later stages to produce general chlorosis.

Iron precipitation in the exodermal root tissues (**50, 53, 55**) is more pronounced in cultures with residual alkalinity than acidity. The slightly greater amounts of iron in the exodermal root tissues of cultures supplied with than without manganese may be of little or no significance concerning

the availability of iron to plants. However, such iron deposits may become subsequently available to plants when high H-ion concentrations or other elements with potentials capable of reducing Fe^{+++} to F^{++} are in the proximity of the rhizosphere (**50**).

The data, in figure 3, depicting amounts of chlorophyll, sap soluble iron or manganese and insoluble iron or manganese (protein bound and precipitated as inorganic salts) in the No. 4 sections of the active D leaves (**48**) of cultures with different iron-manganese ratios supplied with nitrate or ammonium salts, show that the tissues of the nitrate cultures contained,

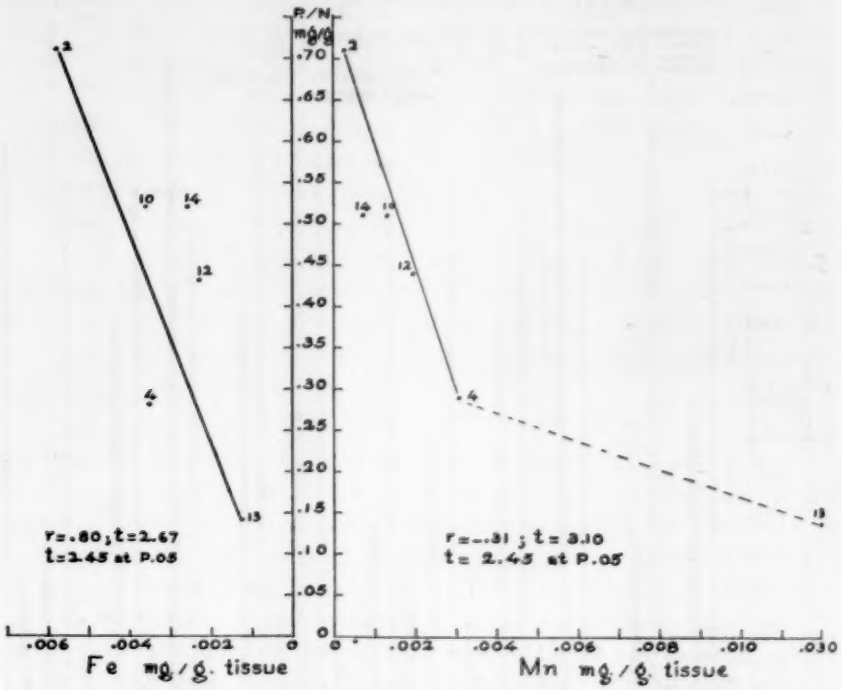


FIG. 4. Protein-nitrogen (P/N) and protein-bound Fe or Mn per gram of fresh tissue in cultures 2, 4, 10, 12, 13, 14.

in general, more manganese than those of the ammonium cultures, whereas iron and chlorophyll were reversed.

Approximately 95 per cent. of the total manganese and 50 to 80 per cent. of the iron in the tissues was insoluble (non-extractable by acetone). Certain tests indicated that precipitated manganese, presumably adsorbed or chemically bound to some colloidal organic matter, could be dissolved in dilute acetic acid solutions.

Manganese was considerably greater in the leaves of cultures 4 and 12, supplied with high-Mn, than in those of 2 and 10, with low-Mn. The differences in the total iron content of the leaves between cultures 2 and 10 or 4 and 12 in favor of 10 and 12, were very small and cannot be com-

pared with similar differences in Mn which were very great between cultures 2 and 4 or 10 and 12. The data clearly indicate that the rate of Mn intake by the roots and translocation to the leaves was considerably greater than that of iron, also, the interference of Mn in Fe absorption by roots and translocation to leaves was very small.

Cultures 13 and 14 with minus-Fe and minus-Mn, respectively, showed small amounts of both elements in the leaves, presumably occurring as contaminations in the C.P. nutrient salts. The Mn-content of the leaves in culture 13, without iron, being greater than in all other cultures with iron might have resulted from the lack of antagonistic effects by manganese or from concentration effects on account of the much smaller plant weights of this than other cultures. Chlorophyll and iron in the leaves correlated with the iron supplies in the nutrient solutions.

The data in figure 4, show that protein-N correlated positively with protein-bound iron but negatively with protein bound manganese. The correlation coefficient for the protein-bound manganese was not as great or statistically as significant as for the protein-bound iron. In culture 13, without iron, the amounts of protein-bound manganese were much higher than in cultures 4 and 12 supplied with equal amounts of manganese but, also, with some iron, suggesting that the presence of iron even in very small amounts prevented appreciable combinations of manganese with proteins.

Mineral nutrients in plant tissues

Potassium.—The amounts of potassium in the tissues of different plant organs, in figure 5, varied in the different cultures, being higher for culture 13 with small plant weights than in all others. The higher potassium concentrations in culture 13 presumably resulted from concentration effects rather than from greater rates of intake of this element by plant roots.

Calcium.—This element, also depicted in figure 5, was slightly higher in the culture with low than high manganese; although the differences in calcium between low and high manganese cultures were not statistically significant.

Kelley (**30**) observed greater calcium concentrations in pineapple plants grown in manganiferous than in non-manganiferous soils, which were thought of later by McGeorge (**37**) as the causes for the development of chlorosis in these plants.

The data in figure 3 and others previously reported (**50, 53, 55**) clearly indicate that manganese as well as calcium absorption is enhanced by NO_3^- but inhibited by NH_4^+. Also, Kelley (**30**) has shown that nitrification is greater in manganiferous than in non-manganiferous soils either because of higher pH in the former than the latter or of a greater rate of nitrate assimilation in the presence than absence of Mn, according to Burström (**12**). Therefore, the formation of NO_3 from nitrogenous organic matter more in manganiferous than non-manganiferous soils and the greater rates

of absorption of Mn and Ca in the presence of NO_3^- than NH_4^+ might explain the greater concentrations of Ca in the plants grown in manganiferous than non-manganiferous soils. McGeorge's (**37**) explanation of manganese chlorosis as resulting from high Ca may not interpret at all the actual conditions in the plant cells.

Magnesium.—The concentrations of magnesium in the plant tissues, in figure 6, were greater in cultures 4, 12 and 13, with high-Mn, than in 2, 10 and 14 with low Mn. The per cent. difference between the high-Mn

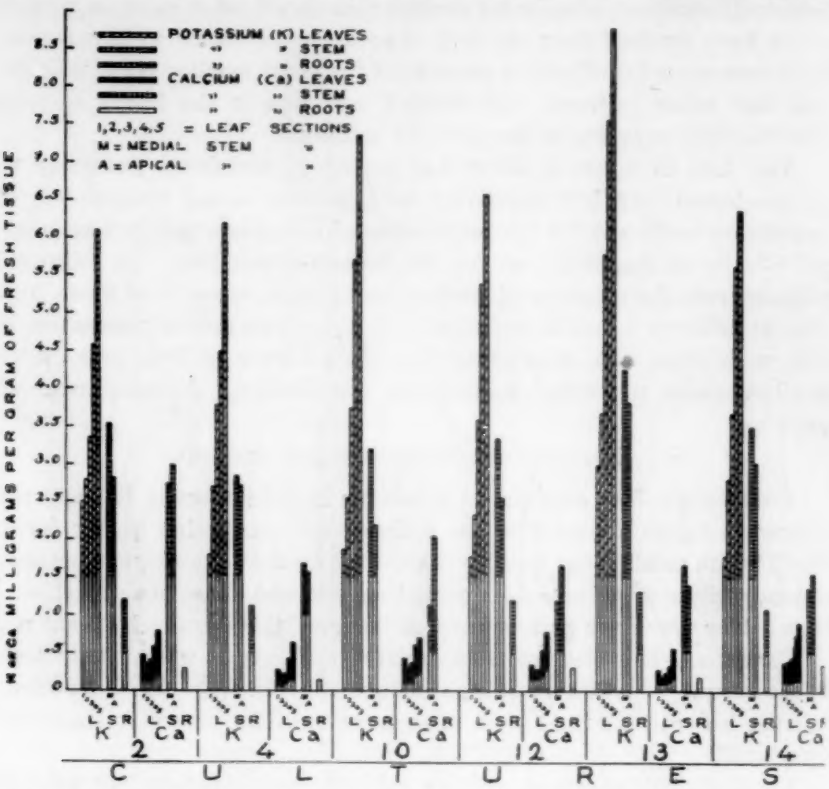

POTASSIUM (K) LEAVES
" " STEM
" " ROOTS
CALCIUM (Ca) LEAVES
" " STEM
" " ROOTS
1,2,3,4,5 = LEAF SECTIONS
M = MEDIAL STEM "
A = APICAL " "
MEQ'S MILLIGRAMS PER GRAM OF FRESH TISSUE
L S R
K Ca
2 4 10 12 13 14
C U L T U R E S

Fig. 5. Potassium and calcium in leaf (L), stem (S), and root (R) fresh tissues.

and low-Mn cultures, namely, 1.6 mg. per gram of fresh tissue equivalent to 24 per cent., was statistically significant at the P .01 level. The greater intake of magnesium by the plants of the high-Mn than low-Mn cultures cannot be explained. Kelley (**30**) obtained similar results in 18 to 24 but not in 5 months old pineapple plants, suggesting that time is an essential factor for such accumulations.

Phosphorus.—Concentrations of this element in plant tissues, in figure 6, were slightly greater for culture 13 than for the others. It is possible that the absence of iron in the solution culture might have rendered phosphorus more available to the roots than in all other cultures supplied with

iron. However, the interpretation offered for potassium, that the higher amounts of this element had resulted from concentration effects on account of smaller plant weights than from higher rates of absorption might apply equally well to phosphorus.

Products of metabolism

Chlorophyll.—Kelley (30) states that microscopic examination of plants grown in manganiferous soils shows fading of the green color in the

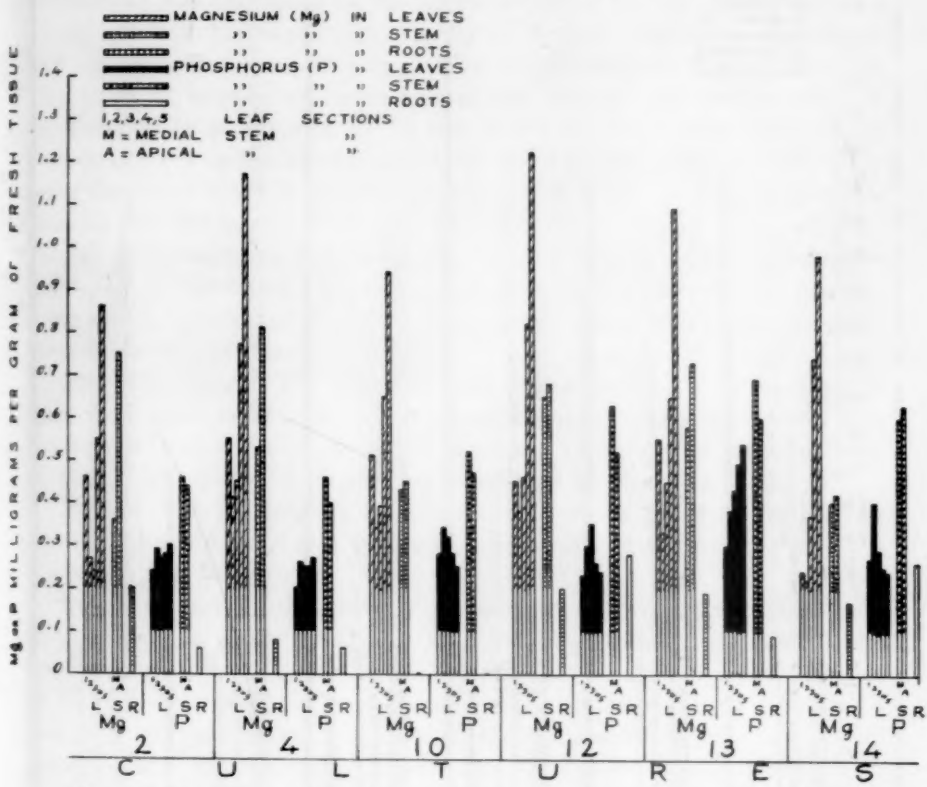


Fig. 6. Magnesium and phosphorus in leaf (L), stem (S) and root (R) fresh tissues.

chlorophyll granules, without their utter disintegration at first, but in successive stages the entire protoplasmic bodies in the cells and all organized arrangement of the protoplasm are broken up and in most advanced stages there is no trace of a granular structure, such as characterizes normal chloroplastids. He concludes that the etiolation of pineapples is caused by some fundamental change affecting the protoplasm and chlorophyll but not the yellow pigments.

The data in figures 3 and 4, the former depicting amounts of chlorophyll and the latter protein and protein-bound iron and manganese, show

that chlorophyll and protein increased with greater amounts of iron in the solution culture but decreased with greater amounts of manganese.

SIDERIS (**57, 59**) found positive correlation between chlorophyll and protein and chlorophyll and iron. The data in figure 4, revealing a similar correlation between iron and protein, suggest that iron is component

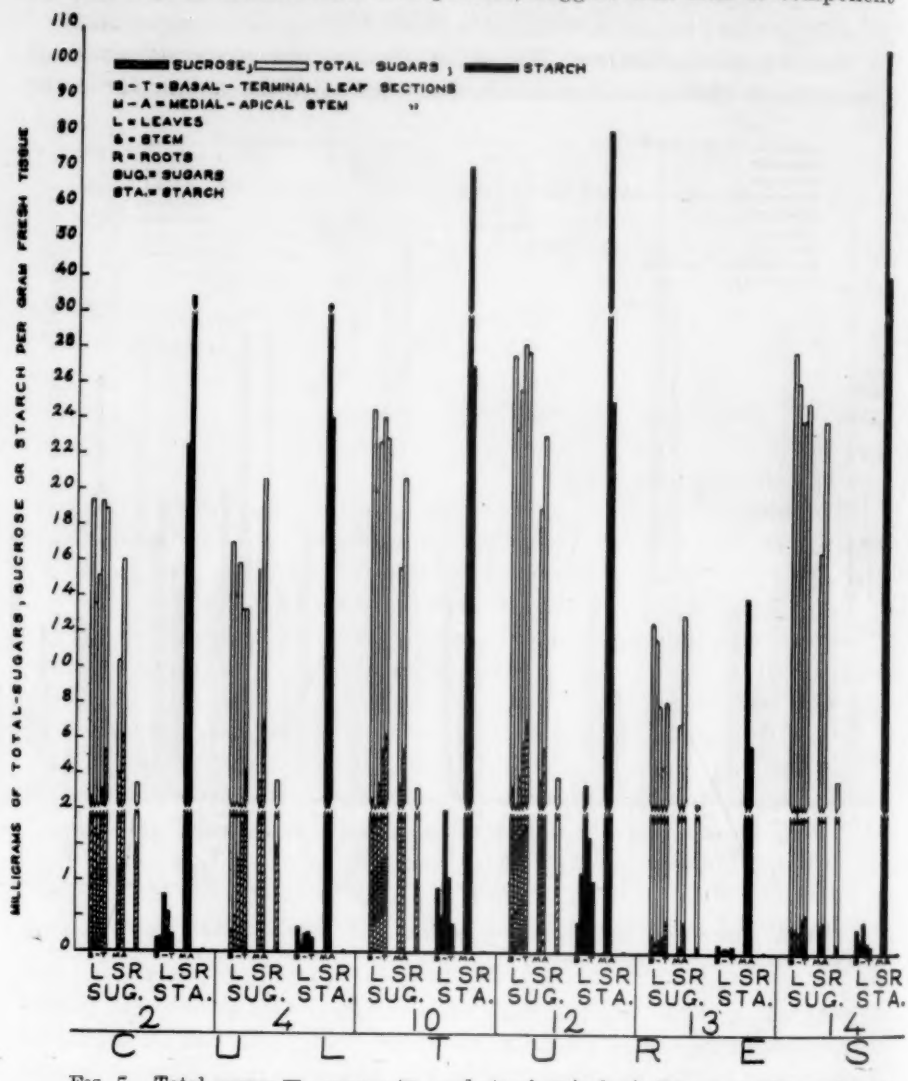


FIG. 7. Total sugars □, sucrose ▨, and starch ■ in leaf (L), stem (S), and root (R) fresh tissues.

of a system directly related to chlorophyll and chloroplastic proteins and that high concentrations of manganese in the cells, especially when iron is very low, affect adversely this system.

SUGARS.—The concentrations of total sugars and sucrose in the tissues,

in figure 7, varied for the different cultures. Total sugar concentrations were greatest in culture 12 and successively in a descending order in 14, 10, 2, 4 and 13. Culture 13, with high-Mn but minus-Fe, had considerably lower sugar than culture 4, also with high Mn but with low Fe (0.05 γ). The difference in sugars between cultures 4 and 13, namely, 41 per cent. in favor of the former and statistically significant at the P .01 level, emphasizes the importance of iron, in concentrations as small as 0.05 γ, in the acceleration of the carbohydrate synthesizing mechanism of plants.

Sucrose concentrations in the leaves depicted by the shaded portions of the columns for total sugars, in figure 7, were greatest in culture 14 and successively in a descending order in cultures 10, 2, 4, 14 and 13. The levels of sucrose concentrations in the various leaf sections were of the same order in cultures 12, 10 and 2, but not in 4 where sucrose was abnormally low in the basal leaf sections No. 1 and increased progressively from the basal No. 1 to the terminal No. 5 sections. Sucrose concentrations in the leaves of cultures 13 and 14 were extremely low, and were related presumably to iron deficiency, in the former, and to manganese deficiency, in the latter. No tests were conducted to associate the low sucrose values in cultures 13 and 14, with hydrolysis or with inhibitive processes in sucrose synthesis.

The differences of the means in total sugars between cultures 2 and 4 were very small and lacked statistical significance. However, similar differences between cultures 10 and 12 or 14 and 13 were very great, in favor of 12 and 14, and were statistically significant at the P .01 level.

STARCH.—In pineapple plants starch is deposited mostly in the stem, figure 7, although some may be found, presumably in a transitory state, in the leaves (**51, 54, 56**).

Starch deposits in the stem were greatest in culture 14 and successively in a descending order in 12, 10, 2, 4 and 13. Similar deposits in the leaves were greatest in culture 12 and successively in a descending order in 10, 2, 4, 14 and 13. The low starch values in the leaves of cultures 13 and 14 might indicate either extremely high amylolytic or very low amylosynthetic activity due, in the former, to iron deficiency and in the latter to manganese deficiency.

The data show positive correlation between sucrose and starch synthesis in the leaves with a coefficient r = .485 significant at the P .01 level. Such correlation might suggest that the conditions in the cell which favored the synthesis of sucrose did, likewise, of starch. The conditions favoring accumulations of both sucrose and starch in the leaves were adequate but not excessive amounts of both Fe and Mn.

Relationships between chlorophyll, on the one hand, and sugars and starch, on the other, were not strictly proportional. In cultures 2 and 4 with as much or more chlorophyll as in cultures 10 and 12 sugars and starch were not as high in the former as in the latter, presumably caused by variations in the physiological status of such plants as a result of dif-

ferences in the iron levels of leaves. GABRIELSEN (**15**) also observed that plants with chlorophyll levels above certain limits did not increase in photosynthetic efficiency if other synergistic factors were lacking.

ASCORBIC ACID.—The amounts of ascorbic acid, in figure 8, with ascending gradients from the basal-nonchlorophyllous to the terminal chlorophyllous sections (**50, 53, 55**) varied for the different cultures. With respect to different groups of leaves, ascorbic acid was highest in the terminal sections of the active D leaves, having lower values in the mature and less active C leaves. Similar values in the young E leaves, were lower than in the D

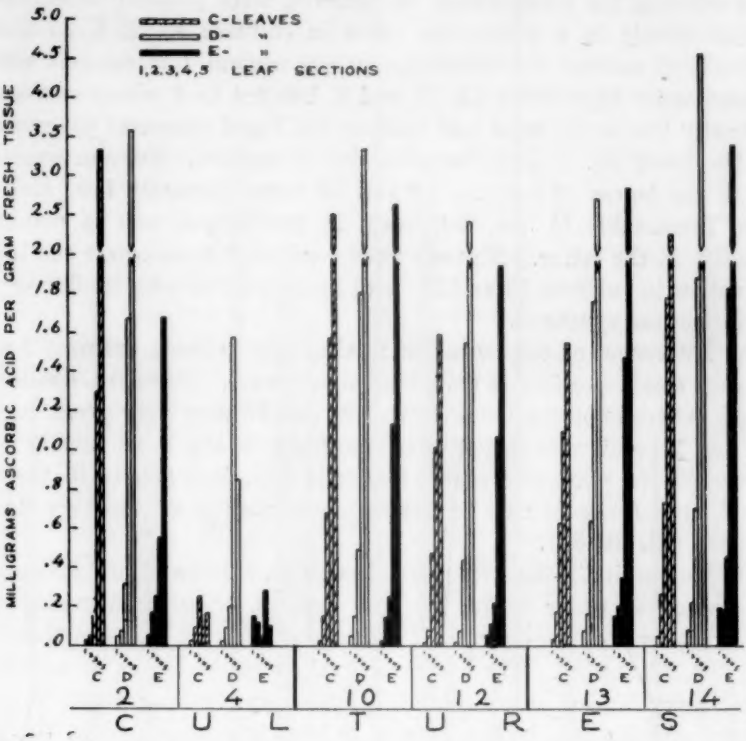


FIG. 8. Ascorbic acid in sections 1, 2, 3, 4, 5 of leaf groups C, D, E.

leaves, because ascorbic acid production, although rising, had not yet attained maximal efficiency.

Cultures 4 and 12 with high-Mn contained less ascorbic acid than cultures 2 and 10, respectively, with low manganese suggesting inhibition of ascorbic acid synthesis or oxidation by high-Mn. High ascorbic acid correlated positively with low manganese in cultures 2 and 10 vs. 4 and 12, respectively, with a coefficient of correlation $r = .61$ and $t = 3.280$, statistically significant at P .01

However, ascorbic acid in culture 13, with high Mn and minus Fe, was considerably higher than in culture 4 also with high Mn but with 0.05 γ

Fe. Moreover, in culture 13, the young E leaves contained more ascorbic acid than the active D or mature C, an anomalous order of distribution of this substance in the various leaf groups. These results suggest that ascorbic acid, like other growth promoting substances, may be utilized in plant growth and its accumulations in the young E leaves of culture 13 had presumably resulted from a lower rate of utilization than of production as attested, also, by the small plant weights of culture 13. The assumption explaining the accumulations of ascorbic acid in the leaves of culture 13 is supported by the findings of SAUBERT-V. HAUSEN (**46**) in peas with removed cotyledons, which showed that Vitamin C alone, in comparison with factors of the Vitamin B complex, biotin and yeast, caused good growth and flowering. Previous studies by SIDERIS, *et al.* (**56**) showed greater accumulations of ascorbic acid in the old B, mature C and active D but not in the young E leaves of low than high nitrogen plants, presumably resulting from a lower rate of utilization of ascorbic acid by the former due to reduced growth activity. Also, the young E leaves of minus-Fe cultures (**51**) contained more ascorbic acid than of plus-Fe cultures, due to reduced growth activity and lower rates of utilization of this substance by the former.

Negative correlation between ascorbic acid and amounts of nitrogen or combinations of nitrogen with potassium, both elements promoting plant growth, are claimed by BRACEWELL, *et al.* (**10, 11**) and WALLACE, *et al.* (**65**) in apples, HAHN and GÖRBING (**21**) in spinach, OTT (**40**), MACLINE and FELLERS (**36**), HAMNER, *et al.* (**23**) and LYON, *et al.* (**35**) in tomatoes, WYND (**67**) in sudan grass, REDER, *et al.* (**44**) in turnip greens, JONES, *et al.* (**28**) in grapefruit and SPEIRS, *et al.* (**62**) in sweet potato. In contrast with the above evidence HESTER (**24**), IJDO (**26**), ISGUR and FELLERS (**27**) and POTTER and OVERHOLSER (**41**) claim positive correlation between amounts of nitrogen and ascorbic acid.

HAMDALLAH (**22**) observed that plants grown without iron or magnesium contained as much or more ascorbic acid as with these elements. HESTER (**25**) found more ascorbic acid in plants supplied with than without manganese. ERKAMA (**14**) reported (his table 17) in pea seedlings somewhat similar results with our data in figure 8; pea plants grown in solution cultures with 100 γ Mn per liter producing smaller weights but containing greater amounts of ascorbic acid than the controls. According to ABERG (**1**), ascorbic acid production is conditioned by a light-independent synthesis, prevailing in sprouting seeds, and a light-dependent synthesis, which is probably connected with the assimilation of carbon dioxide. The same investigator, also, claims that ascorbic acid being translocateable from leaves to stem and roots, its amounts may depend on rates of translocation.

High light intensities, moisture deficiencies and high topographic elevations, presumably related adversely to plant growth, were observed to increase the ascorbic acid content of tissues (**8, 31**).

BONNER and BONNER (**9**) and DENNISON (**13**) consider ascorbic acid an essential factor in plant growth which is in agreement with our views and others cited above. Therefore, higher accumulations of ascorbic acid in the tissues of plants growing at lower than higher rates indicate non-utilized quantities in plant growth processes rather than greater rates of ascorbic acid synthesis by plants with lower rates of growth. JONES, *et al.* (**28**) have the same point of view of ascorbic acid accumulations in their diagram on possible factors related to the formation and utilization of ascorbic acid.

Discussion

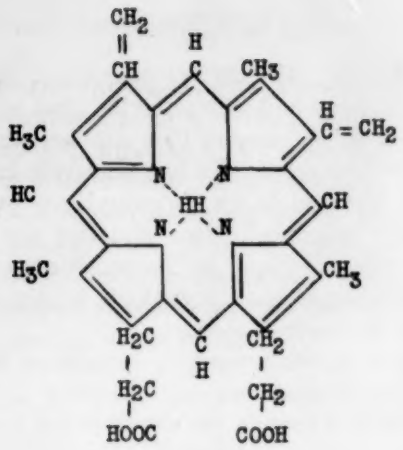
The above results, disclosing that the chloroplastic protein-chlorophyll system may be affected either favorably or adversely by different iron-manganese ratios, suggest that iron may be a component part of this system and the presence of manganese in the chlorophyllous cells in greater amounts than required presumably antagonizes the assimilation of iron by this system or interferes with the physiological functioning.

GRANICK (**18**) investigating the brown color of the cells of an x-ray mutant of *Chlorella vulgaris* found protoporphyrin 9 to be a metabolic precursor of chlorophyll. Protoporphyrin 9 has the basic plan of the ring structure and similarities of the side chain patterns of chlorophyll with Fe instead of Mg atom attached to the four N atoms of the pyrrol ring.

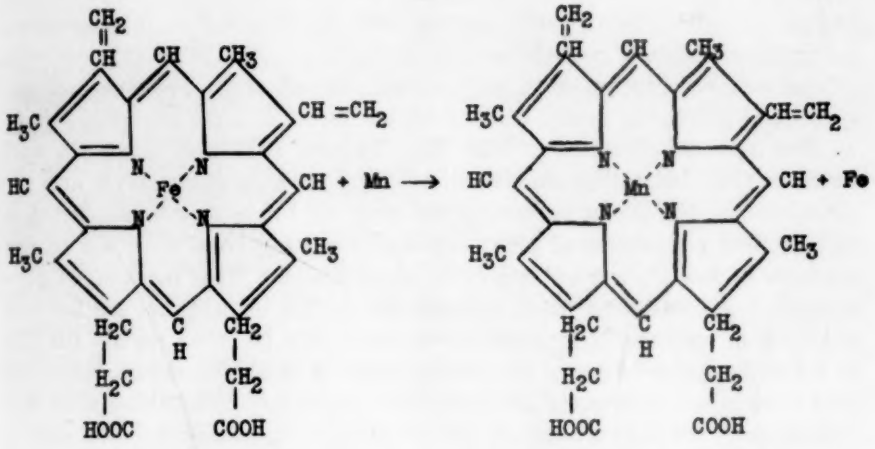
The relation of protoporphyrin 9 to chlorophyll, although shedding light on the role of iron in the synthesis of chlorophyll, does not explain satisfactorily the physiological mechanism of Mn in the development of chlorosis.

Porphyrins are basic structural units of the cytochromes, peroxidases, oxidases, etc., in addition to chlorophyll and, consequently, intimately associated with many essential physiological processes in the cell. They may undergo, *in vitro,* replacement of the Fe atom by other chemically related elements, according to TAYLOR (**63**), and exhibit similar or different properties. The preparations, in the laboratory by GJESSING and SUMNER (**17**), of the metalloprophyrins of Co, Ni, Mn and Cu, by substitution for Fe in the hemin from defibrinated cows' blood, were inactive except the manganese protoporphyrin which had 20 to 30 per cent. as much peroxidase activity as the iron protoporphyrin.

BAUDISCH (**4**) explains the physiological activity of the metal atom in the pyrrol ring as due to paramagnetic properties generated by the arrangement of a positive ion (Fe, Mn or Cu, etc.), in the center, surrounded by neutral or negative atoms (N) which tend to gain electrons by coordinating atoms, forming functional units in definite energy stages of electric or magnetic fields. Hence, any changes in the structure of porphyrins, which may result from replacement of Fe by Mn or other metals, might either increase or decrease the activity of the prosthetic substance, be it an enzyme or chlorophyll, and alter the rhythmic operation of processes on which depend most cell functions.

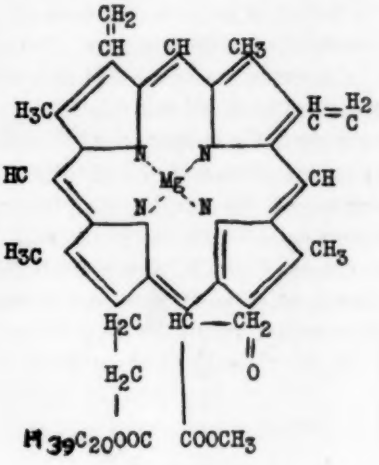


Protoporphyrin



Iron Protoporphyrin

Manganese Protoporphyrin



Chlorophyllα

The findings of GRANICK (**18**) that protophyrin 9 is the precursor of chlorophyll, and of GJESSING and SUMNER (**12**) that substitutions of Mn for Fe, *in vitro*, are possible, suggest that similar substitutions may be possible in cells with extremely low-Fe but relatively high-Mn concentrations. GRANICK (**19**) reported lately that vinyl groups are necessary in protoporphyrins for the insertion of iron and that the latter is inserted only in completed porphyrin rings, by Haemophilus organisms, making implausible any consideration that porphyrin might be synthesized in piecemeal fashion around the iron atom.

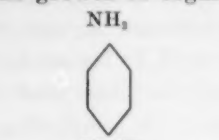
Our data in figure 4 showing positive correlation between protein-N and iron and negative correlation between protein-N and manganese suggest possible association of a protein prosthetic group containing iron, the formation of which and that of the associated protein are limited in the presence of very small amounts of iron and high ones of manganese. There are many known proteins and enzymes in association with prosthetic groups containing iron or other metals, such as haemoglobin, chlorocruorin, helicorubin, catalase, peroxidase and cytochrome, with iron, turacin with copper and chlorophyll with magnesium. In all these proteins and enzymes the prosthetic group containing the metal is a porphyrin.

The findings of GRANICK (**18, 19**), TAYLOR (**63**) and GJESSING and SUMNER (**12**) indicating conversion of porphyrins to chlorophyll and replacement of the metal in the pyrrol ring by other metals suggested a hypothetical mechanism of plant chlorosis resulting from high Mn in the presence of low Fe concentrations in plant tissues. This mechanism presumably associated with plant chlorosis due to Mn or other metals but not to nitrogen, may result from substitutions of Mn or other metals for 2H or Fe in the pyrrol ring of the protoporphyrin molecule, shown below, in cells containing inadequate amounts of Fe necessary to combine with the continuously forming protoporphyrin molecules necessary for the subsequent synthesis of chlorophyll. It may be further assumed that chlorophyll synthesis, if effected by substitution of Mg for Fe in protoporphyrins, releases protoporphyrin bound iron which is successively replaced by Mg and allowed to combine with newly forming protoporphyrin molecules. Thus, iron performs, in accordance with this assumption, the role of a catalyst which is in agreement with all theories heretofore advanced.

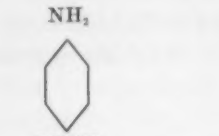
In line with the above thought, MILLIKAN (**39**), discussing the evidence obtained recently from experiments which indicated that the inhibition of symptoms of lower leaf scorch in flax by molybdenum was presumably caused by high manganese concentrations in the soil, states, also, that an excess of Mn, Zn, Co, Cu or Ni will induce iron deficiency chlorosis in plants. However, no explanation is offered on the nature of the antagonism between Mo and Mn whether resulting from the repelling forces of the ions Mn^{++} vs. Mo^{++} and MnO_4^{--} vs. MoO_4^{--} or from precipitation of Mn^{++} by MoO_4^{--}.

Replacement of Fe by Mn in protoporphyrins may produce physiolog-

ically inactive substances similar to those discussed by WOOLLEY (**66**) in his review of biochemical antagonisms where the substitution in a basic chemical structure, protein or vitamin, of a prosthetic unit for another changes completely the physiological activity of said structure. For example, the inactivation of p-aminobenzoic acid by sulfonilamide, an analogue of the former in which the carboxyl group is replaced by sulfonamide group, shown below, is effected by the sulfa drugs displacing p-aminobenzoic acid from its combination with specific proteins and thereby inactivate enzymes important in the growth of organisms.

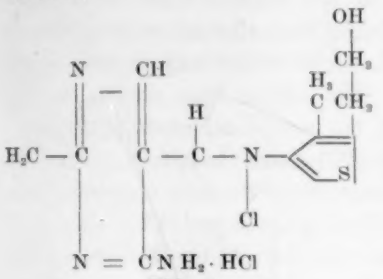


p-amino benzoic acid

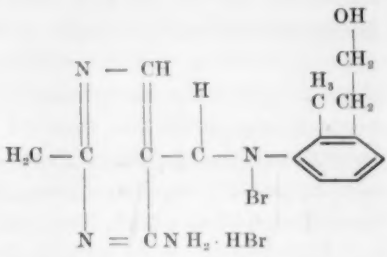


Sulfonilamide

Also, a similar case of biological antagonism is the inactivation of thiamin by pyrithiamin, an analogue of thiamin, in which the thiazole ring is replaced by a pyridin ring, or, more specifically the S by — CH = CH —, as follows:



Thiamin



Pyrithiamin

The biochemical antagonism of Mn vs. Fe may be in many respects analogous to the antagonisms, mentioned above, of sulfa drugs vs. p-aminobenzoic acid or of pyrithiamin vs. thiamin.

The data, in figure 1, showing that plant weights increased progressively with the amounts of Mn from 0.05 to 5.0 γ, suggests certain beneficial or synergetic effects of Mn. Such effects, pertaining to agriculture, in general, were reviewed by MCHARGUE (**38**).

Investigations on the physiological functions of manganese indicate that it is an activator of enzymes. ADLER *et al.* (**2**) observed that Mn and Mg but not Ca, Zn or Cd activated coenzyme II for the oxidation, by the isocitric enzyme, of isocitrate to α Keto-β-carboxylglutarate; a reaction of paramount importance in plant metabolism, because it associates the function of the isocitric dehydrogenase system as a link between carbohydrate breakdown and protein synthesis as follows: carbohydrate ⟶ isocitrate⟶ α Ketoglutarate + NH_3⟶ α-iminoglutarate ⟶ glutamate. SHILS and MCCOLLUM (**24**) found that Mn-deficient rats con-

tained considerably reduced arginase activity in their liver, and were sterile. LIPMAN (**33**) states that Mn or Mg are essential factors for the catalysis of pyruvic acid dehydrogenation with oxygen as hydrogen acceptor. Both Mn or Mg, according to the same author (**33**), are essential components of the carboxylase system of Lohmann, whereby the function of thiamin is to catalyze the decarboxylation of pyruvic acid to acetaldehyde and CO_2.

Studies on the physiological functions of Mn in plants have not been as extensive as in animals. BURSTRÖM (**12**) and LUNDEGARDH (**34**) obtained information indicating that Mn accelerates the rate of respiration of and intake of nutrients by roots. BERTRAND (**6, 7**) has associated manganese with the composition of related oxidizing enzymes, such as laccase and oxidase.

The allegation that Mn favors the intake of mineral nutrients by roots from solution cultures or soil deserves greater substantiation as to quantities of Mn in various substrata and conditions of plant growth. KELLEY (**30**) claims that more lime (CaO) was taken up by pineapple and other plants from manganiferous than non-manganiferous soils without making allowance for the greater lime content of the former than latter soils. Manganiferous soils, because of their colluvial and alluvial origin, contain greater amounts of water soluble mineral elements and may be less acidic than adjacent non-manganiferous soils. The higher base status or percentage base saturation resulted in many but not in all cases from lower rainfall and less leaching of bases. KELLEY (**30**) shows that average per cent. composition of 10 manganiferous and 9 non-manganiferous soils, respectively, was: K_2O, 0.81 and 0.56; Na_2O, 0.37 and 0.28; CaO, 0.53 and 0.30; MgO, 0.45 and 0.40; Mn_3O_4, 4.95 and 0.35; Fe_2O_3, 22.2 and 26.0; Al_2O_3, 16.93 and 13.6; P_2O_5, 0.19 and 0.09; SO_3, 0.20 and 0.21; TiO_2, 0.87 and 2.22, etc. These figures clearly indicate that the greater uptake of Ca by plants from manganiferous than from non-manganiferous soils should be attributed to the greater Ca content of the former than the latter soils rather than to the influence of Mn. Nitrification, which is higher in manganiferous than non-manganiferous soils, might enhance the uptake by roots of more Ca^{++} from the former soils, because NO_3^- favors whereas NH_4^+ inhibits the absorption of both Ca^{++} and Mn^{++}.

Plant weights, sugars or starch and chlorophyll, all three or four presenting a chain of interdependent links, were best in cultures 7, with 5.0 γ Mn and 0.5 γ Fe, and in 11 with 5.0 γ Mn and 5.0 γ Fe. Such concentrations, although ranging in iron-manganese ratios from 1:10 to 1:1, respectively, were satisfactory because they were outside the deficiency or toxicity limits for Mn or Fe.

Manganese toxicity resulting in cultures with very low but not moderate amounts of iron may cause growth stunting or death to plants treated with high concentrations of this element. Eight of the plants in culture 4 and fourteen in culture 13 of a total number of 16 plants per culture died

at or before floral differentiation, and the fruits produced by the surviving plants were small and of poor quality. In manganiferous soils, where iron limitations are not as great as in solution cultures, plants may not die but produce fruits which often are shell chlorotic and split before or at ripening.

Ascorbic acid being higher in cultures 2 and 10 with 5.0 γ Mn and not in 4 and 12 with 50.0 γ Mn suggests possible partial inhibition of the formation of this substance under high Mn conditions. However, in culture 13, with equal amounts of Mn as in 4, ascorbic acid was high, which was contrary to our expectations, but this condition was explained as resulting from a decreased rate of utilization in plant growth which is evidenced by the small plant weights of this culture.

In previous studies (**58**) comparable results, as with ascorbic acid, were obtained in the utilization of sugars for plant growth; small plants containing greater concentrations of sugars due to lower rates of utilization than large plants. Under optimal conditions of plant growth the amounts of ascorbic acid in the leaves presumably should correlate with the sugars because the synthesis of the former depends on the latter. But under adverse conditions where growth and carbohydrate synthesis are interrupted, as in culture 13, the production of ascorbic acid from sugars, may continue to operate, whereas growth which depends on the utilization of both sugars and ascorbic acid, may stop and certain substances, such as ascorbic acid, accumulate.

Ascorbic acid accumulations under adverse growth conditions are associated more with cultures supplied with nitrate than ammonium salts as sources of nitrogen. For example, ascorbic acid accumulated more in the minus-Fe than in the plus-Fe cultures of the nitrate series, whereas, the levels of ascorbic acid, in the ammonium series, were approximately equal for the minus-Fe and plus-Fe cultures. Also plant weights in the latter series were approximately equal for both cultures. Although such results suggest that ascorbic acid might take part in nitrate reduction in the chlorophyllous tissues of pineapple leaves where the former is very plentiful and the latter disappears rapidly presumably after reduction and subsequent assimilation under optimal light and temperature conditions, the experimental evidence is more in favor of the utilization of ascorbic acid in growth than in nitrate reduction. However, certain preliminary tests, *in vitro*, employing leaf sap, ascorbic acid KNO_3 and toluene, showed in the course of 12 hours little or no nitrate reduction, but very pronounced reduction in the course of a week. These tests, although undecided, might have yielded different results had the conditions for the ascorbic acid-nitrate oxido-reduction system been better understood.

Summary

Pineapple plants grown in solution cultures with different concentrations of iron (0.05, 0.5 and 5.0 γ) and manganese (0.05, 0.5, 5.0 and 50.0 γ)

in various combinations and supplied with equal amounts of all essential elements and nitrogen as nitrate- or ammonium-salts developed under the various conditions, as follows:

1. Most satisfactory growth, as measured by plant weights, was obtained in culture 7 with 0.5 γ of Fe and 5.0 γ of Mn. The cultures with 0.05 γ Fe and 0.05 γ Mn or with 0.05 γ Fe and 50 γ Mn produced smaller plant weights presumably of Fe and Mn deficiency for the former or Mn toxicity for the latter. The plants of cultures minus-Fe failed to produce fruits and most died.

2. The concentrations of both iron and manganese increased in the tissues in proportion with the amounts in the nutrient solution of the cultures supplied with nitrates. In the cultures supplied with ammonium similar concentrations of Mn in the tissues were almost one half as great as those of the nitrate cultures, due presumably to antagonism between Mn^{++} and NH_4^+. Iron concentrations were greater in the tissues of the ammonium than of the nitrate cultures.

3. Certain amounts of iron and manganese in the leaf tissues were in combination with definite protein fractions; the amounts of protein increasing with iron but decreasing with manganese.

4. The tissues of the high-Mn cultures contained more magnesium than of the low-Mn cultures; but other elements, such as potassium, calcium and phosphorus were taken up from the nutrient solution at approximately equal rates by high-Mn or low-Mn cultures.

5. Sugars and starch were high in cultures with good growth and ample chlorophyll, but the latter was not proportionally related to the former in all cultures.

6. In cultures supplied with iron, ascorbic acid was higher in those with low-Mn than with high-Mn. But in the minus-Fe and with high-Mn cultures ascorbic acid accumulated in the young leaves. This condition was explained as resulting from lower rates of utilization due to decreased rates of plant growth.

7. The development of plant chlorosis from high manganese is explained by the hypothesis of biochemical antagonism of homologous substances, whereby manganese is presumably substituted for iron in protoporphyrin 9, the chlorophyll precursor, thereby inactivating the latter for subsequent conversion to chlorophyll.

PINEAPPLE RESEARCH INSTITUTE
UNIVERSITY OF HAWAII
HONOLULU, T. H.

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AUTONOMIC DIURNAL CYCLES IN THE WATER RELATIONS OF NONEXUDING DETOPPED ROOT SYSTEMS

ROBERT M. HAGAN

(WITH ELEVEN FIGURES)

Received November 12, 1948

The intake of water through the freshly cut end of the stumps of previously wilted detopped plants has received little study. The term "negative exudation" will be used to refer to this reverse flow of water through the cut stump into the root system. This terminology used by McDERMOTT (**10**) appears to avoid the confusion which may arise if such uptake of water is referred to as "absorption" by the root system.

ROSENE (**12**), EATON (**4**), and others have investigated the effects of solution concentrations on reversing the movement of water through root systems. LITVINOV (**9**), CRAFTS and KENNEDY (**3**), KRAMER (**8**), and later McDERMOTT (**10**) have examined some aspects of the influence of the moisture content of the soil surrounding the roots on water intake by the cut stump of detopped plants. However, no measurements of negative exudation extending over periods of several days have come to the writer's attention.

Methods

Twelve-week-old field sunflower (*Helianthus annuus*) plants, grown in small containers under the normal day-night environment of a greenhouse, were brought into a controlled environment room wherein all experiments were carried out under constant illumination and at a temperature of 28° C ± 1°.

The plants were allowed to reduce the soil-moisture content to the permanent wilting percentage. While wilted they were detopped under water and as quickly as possible connected by means of tightly fitting rubber tubing to a series of pipettes which permitted measurements to be made of water movement into the stump and provided a convenient means of replenishing the water supply. The arrangement of this apparatus is shown in figure 1.

The plants were grown in cans 4 inches in diameter and 6½ inches high. The openings in the lids through which the plant stems passed were sealed by wrapping the stem with elastic rubber tape and coating the tape with paraffin.

In some experiments the composition of the soil atmosphere around the roots was modified by introducing nitrogen or carbon dioxide supplied from compressed gas cylinders. These gases were first humidified and then passed through small bubble-counter bottles which provided visual checks of the rate of gas flow into each can. The gases entered at the top of the

can through a second hole in the lid and escaped at the bottom through a vent made of ¼-inch OD tubing covered with an 80-mesh screen and a ¼-inch layer of sieved sand (1 to 0.5 mm. fraction). The soil rested on the sand layer which facilitated the circulation of gases. The gases passing from the vent were periodically analyzed for oxygen content using a Pauling Oxygen Analyzer.

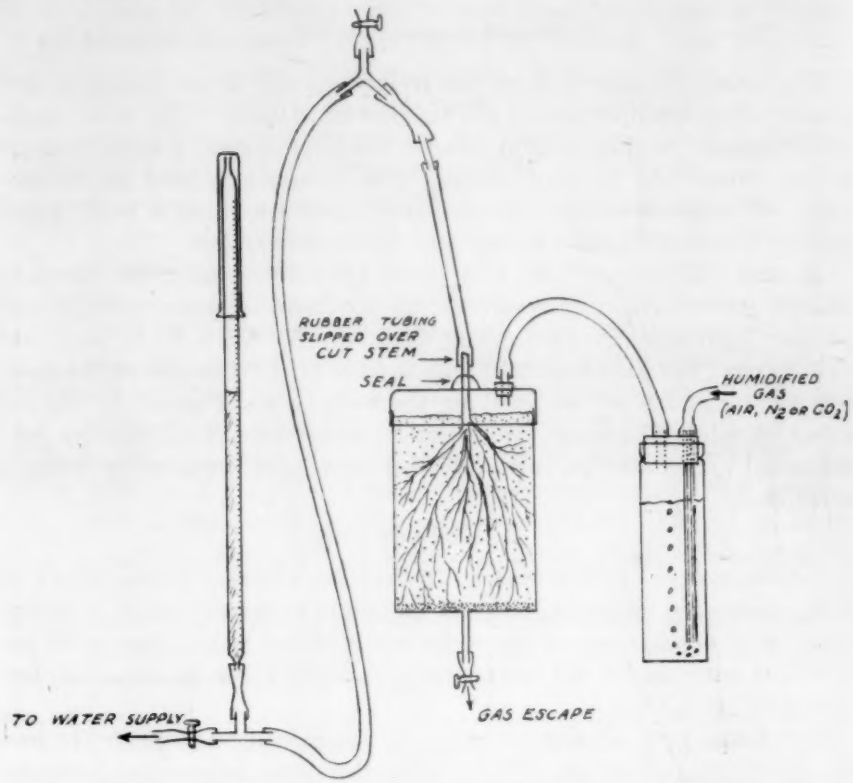

FIG. 1. Diagram of essential parts of one unit of the apparatus for measuring negative exudation. The can contains the root system of a detopped plant which is connected by rubber tubing to the water supply in the inverted pipette. The pipette may be raised or lowered to keep the water surface in the pipette level with the top of the can, and the pipette may be refilled when empty by opening the clamp in the water supply line. To study the effects of aeration on negative exudation, the atmosphere around the roots may be controlled by passing humidified air or gases into the can through a bubble-counter bottle which provides a check on the rate of flow. The apparatus is housed in a constant environment room.

The water surfaces in the pipettes were frequently reset at the level of the lids of the soil cans to avoid the development of appreciable positive or negative hydrostatic heads on the root systems. The Y-tube and clamp in the line above each detopped stump permitted air or gas bubbles occasionally trapped or formed in the line to be bled out of the system.

Results and discussion

Water intake by the cut stumps, or negative exudation, proceeded at a high rate initially and approached zero after approximately one week. In

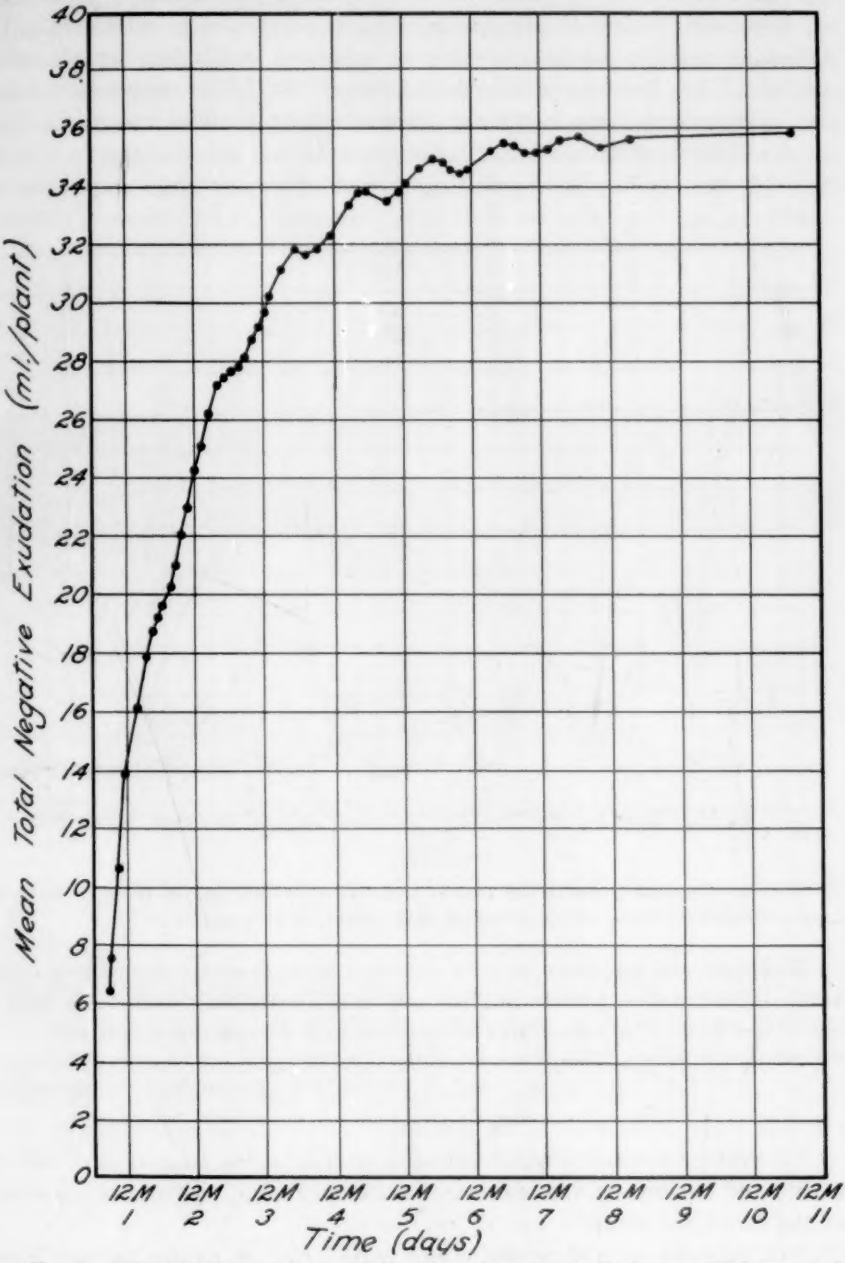

FIG. 2. Mean total negative exudation for three plants during an 11-day period. Note that 12M refers to 12 midnight.

figure 2 the mean total negative exudation for three plants has been plotted against time. Examination of this curve leads to the very interesting observation that negative exudation by living roots follows a distinctly periodic function.

The cyclic nature of negative exudation is depicted more clearly in the following figures where the rate of negative exudation, expressed as ml./min. $\times 10^3$, has been plotted against time. Rate-time curves for aerated root systems are shown in figures 3 and 4. This periodic function is characterized by maxima occurring near midnight and minima near noon. In figure 5 data for a plant exhibiting a cycle of a very large amplitude are given.

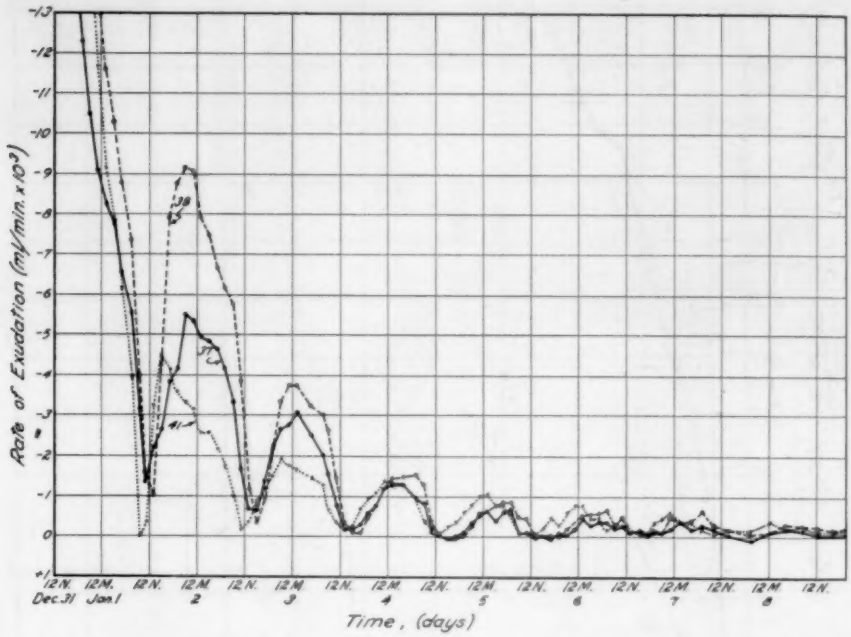

FIG. 3. Diurnal cycles in the rate of negative exudation by the roots of three detopped sunflower plants. Note M refers to midnight, N to noon.

This periodic behavior may be detected as soon as the extremely rapid water uptake which occurs immediately after detopping tapers off, and it continues for six or more days with gradually decreasing amplitude.

Inasmuch as these experiments were carried out under constant environment conditions, the observed cycles in the rate of negative exudation must be truly autonomic. The gradual decrease in the amplitude of these cycles with time would suggest that this periodicity is related to metabolic activity which diminishes with time as the available respiratory substrate of the roots is depleted.

Fluctuations in root pressure and in the rate of exudation have been observed for many years, and their dependence upon variations in the en-

vironment often debated. SPEIDEL (**14**) has reviewed much of this work. GROSSENBACHER (**5, 7**) and Speidel, working independently, both report that in *Helianthus annuus* exudation follows a well-defined autonomic 24-hour cycle with maxima occurring approximately at noon and minima at midnight. Grossenbacher found the timing of this cycle to be dependent upon the environmental conditions prevailing during the growth period.

A comparison of the results obtained by Grossenbacher and by Speidel on positive exudation with the present work on negative exudation indicates that positive and negative exudation follow a similar cycle with the maxima and minima exactly 12 hours out of phase. These investigations strongly suggest that the periodicities in both positive and negative exuda-

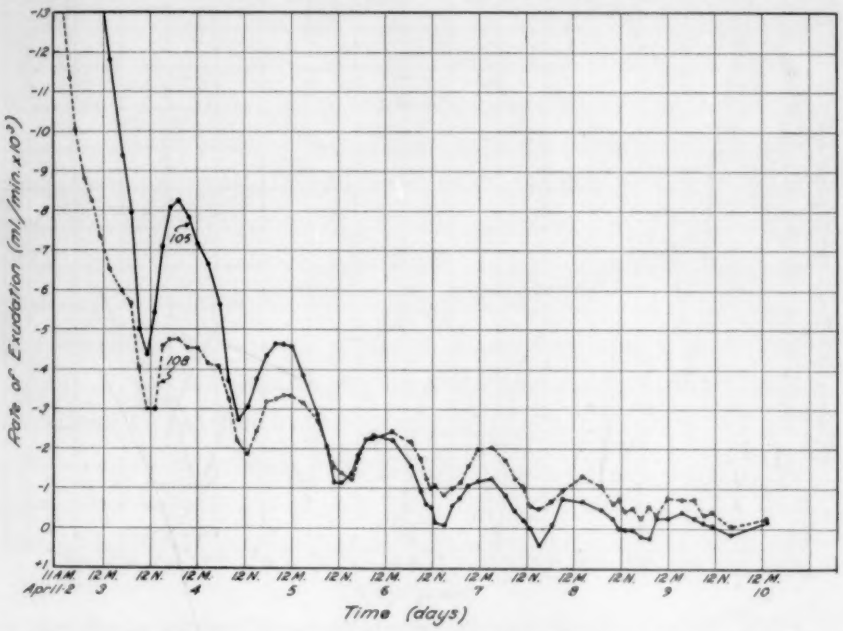

FIG. 4. Diurnal cycles in the rate of negative exudation by the roots of two detopped sunflower plants. Roots continuously aerated. Note M refers to midnight, N to noon.

tion arise from the same root activities and that the cycles observed in negative exudation are an extension of the same cyclic behavior observed in positive exudation by Grossenbacher and by Speidel. For example, the data for the root system plotted in figure 5 show on April 3 a distinct cycle in negative exudation. On April 4 and on the following days, the cycle includes positive exudation near noon and negative exudation around midnight. During these days the quantity of positive exudate approaches that of the negative exudate, indicating that the root has reached an equilibrium condition with little net gain or loss of water. These data demonstrate the existence of a rhythmical behavior in root systems even in the absence of positive exudation.

No satisfactory explanation for the diurnal cycles in exudation is apparent. SKOOG, BROYER, and GROSSENBACHER (**13**) were unable to detect a periodic liberation or removal of osmotically active material within the root by analyses of exudate. These workers found that the effectiveness of auxin treatments in increasing the rate of exudation also follows a diurnal cycle and, on the basis of these findings suggest that there is a relation between auxin and the mechanism responsible for the diurnal cycles in exudation. However, they were unsuccessful in an attempt to correlate the increase in exudation due to auxin treatment and the diurnal periodicity with changes in respiratory activity, but they point out that such a

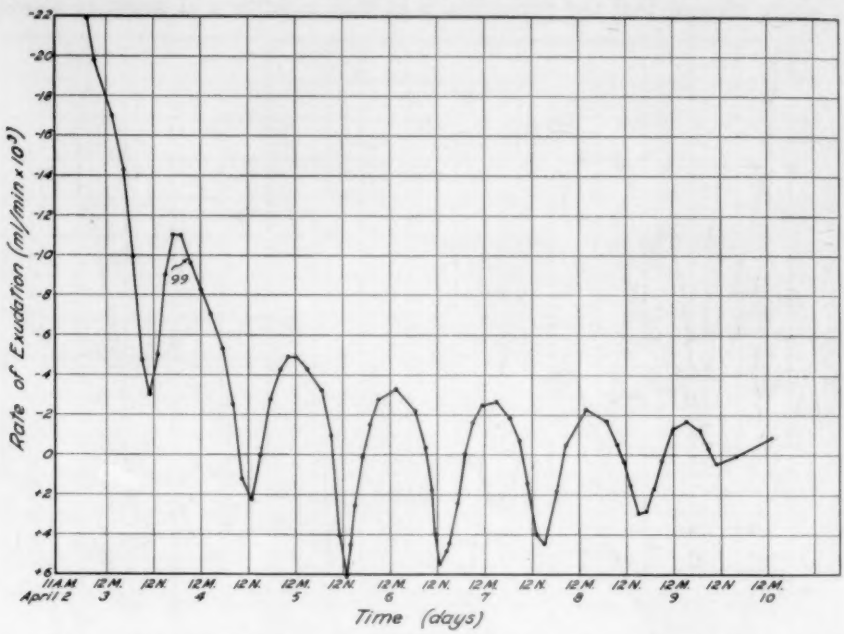

FIG. 5. Diurnal cycles of large amplitude in the rate of negative exudation by the roots of a detopped sunflower plant. Roots continuously aerated. Note M refers to midnight, N to noon.

correlation may be demonstrable under other conditions. A day-night rhythm in the rate of carbon dioxide production by excised roots in which the respiratory activity appears to proceed at a higher rate during the daytime hours than during the night appears in a graph by WHITE (**15**). Unfortunately no data are given nor are any comments or explanations of the graph presented. SPEIDEL (**14**) suggests the possibility that the supply of respiratory material is subject to periodic fluctuations for unexplainable reasons, or that, if the supply of respiratory substrate is constant, the enzymatic activity responsible for the conversion of this material to energy fluctuates rhythmically. In consequence of this, there is a periodic increase and decrease in turgor and corresponding expression or absorption of water by these cells.

In view of the suggested dependence of exudation periodicity on metabolic activity, it is at once of interest to examine the effects of aeration on the maintenance of periodicity. Such a study cannot be carried out on a positively exuding system inasmuch as exudation itself depends on continued aeration. Now that negatively exuding roots are seen to show the same periodic behavior as positively exuding roots, negatively exuding roots offer a means of examining aeration as a factor in periodicity. The effects of saturating the atmosphere around the roots with nitrogen and carbon dioxide are shown graphically in figures 6–9.

In the experiment shown in figure 6, high purity nitrogen, bubbled

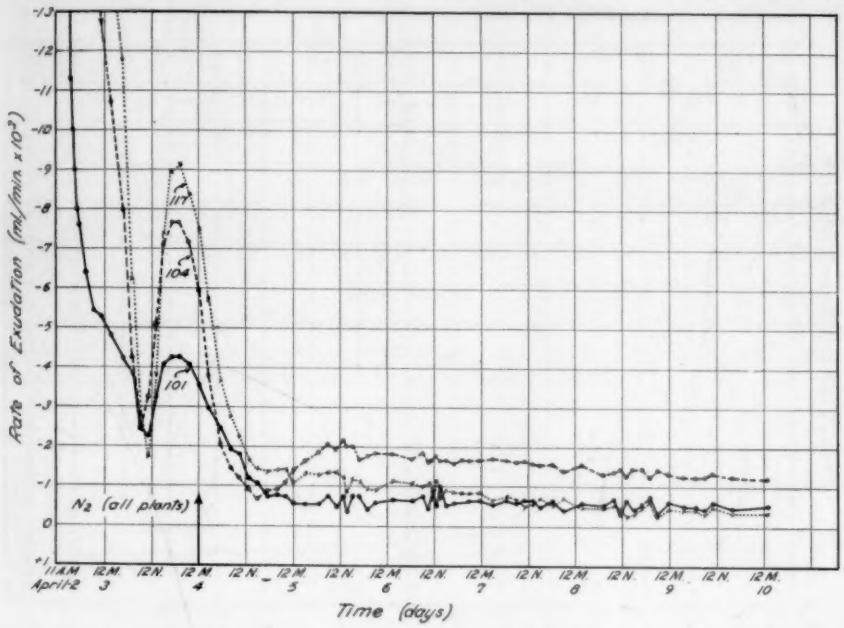

FIG. 6. Termination of cycles in the rate of negative exudation by sunflower roots following introduction of nitrogen at 12 midnight, April 3.

through aqueous alkaline pyrogallol solution to remove any traces of oxygen, was introduced beginning at 12 midnight, April 3, and passed continuously through the container. Removal of oxygen from the system resulted in an immediate termination of the diurnal cycles, while cycles in the aerated checks of the same group of plants continued normally (cf. figures 4 and 5). Previous work had shown that incomplete removal of oxygen by nitrogen did not affect the cycles. Accordingly, it may be concluded that the periodic cycles will continue although the oxygen content of the surrounding atmosphere is appreciably reduced, but this behavior is quickly stopped when roots are deprived of oxygen.

Carbon dioxide was found to be more effective than nitrogen in terminating the cyclic behavior. Incomplete saturation with carbon dioxide

was sufficient to stop the cycles immediately (see figures 7–9). In analyzing the results of the carbon dioxide treatments, it must be realized that the introduction of carbon dioxide was combined with a reduction in the oxygen concentration. However, as one might expect in view of the well-known narcotic effect of carbon dioxide on plant tissue, carbon dioxide was considerably more effective in stopping the periodicity than the simple removal of oxygen by the introduction of nitrogen.

Several attempts were made to determine whether the effect of carbon dioxide on periodicity was reversible. Removal of the carbon dioxide by air apparently failed to restore a marked periodicity (see figures 8 and 9), but this matter requires further investigation.

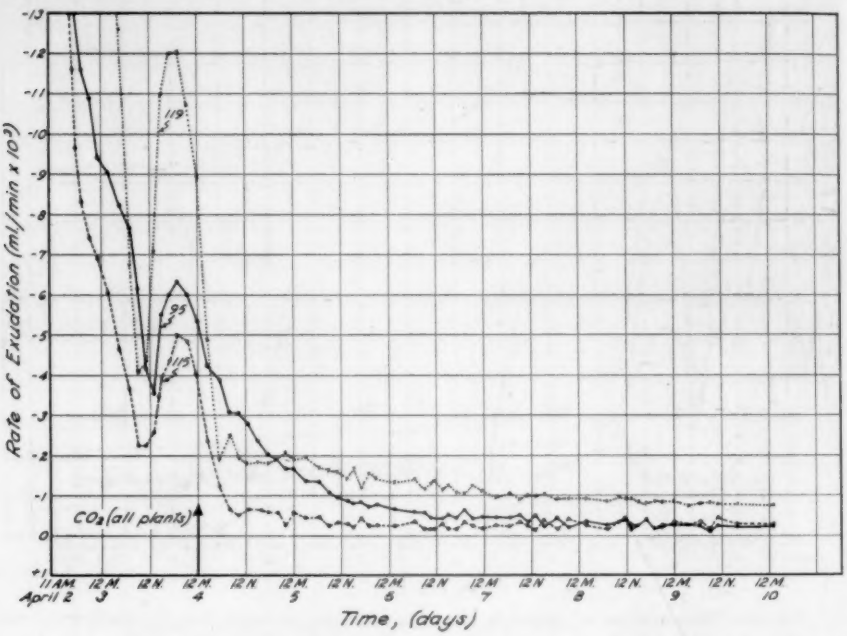

FIG. 7. Termination of cycles in the rate of negative exudation after introduction of carbon dioxide at 12 midnight, April 3.

The termination of these cycles by nitrogen and by carbon dioxide, while cycles continued in the aerated roots, proves that these diurnal cycles are not an artifact dependent upon some unrecognized cyclic change in the environment. GROSSENBACHER (**6, 7**) has also presented strong evidence that these cycles are truly autonomic.

Skoog and co-workers consider the possibility that periodicity may arise as a result of changes in permeability. However, they add that this suggestion suffers from several difficulties. It is assumed that these writers have in mind the idea that changes in permeability would control the rate at which water could be absorbed from the soil, pass through the root, and appear as exudate. The finding that the maxima in the cycles for positive

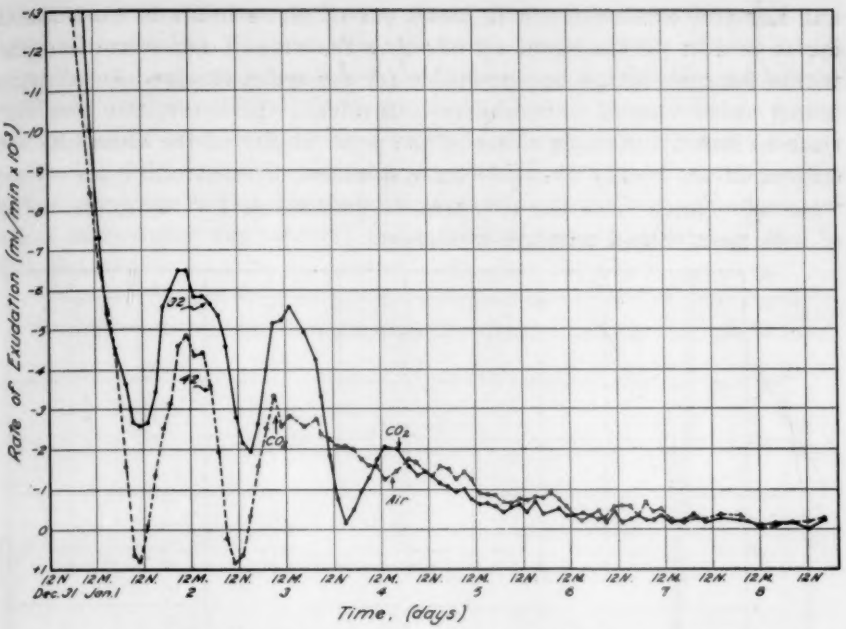
Rate of Exudation (ml./min. x 10⁻³)
O2
N2
CO2
CO2
Air
12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N.
Dec. 31 Jan. 1 2 3 4 5 6 7 8
Time, (days)

FIG. 8. Rapid cessation of cycles in the rate of negative exudation following introduction of carbon dioxide for short periods beginning at times indicated by small arrows. Carbon dioxide around roots replaced by air at times indicated.

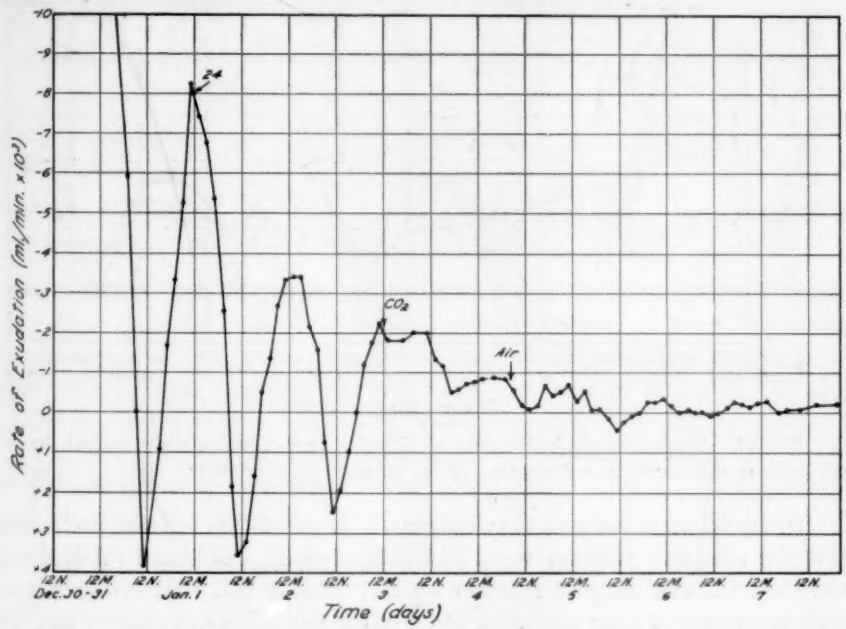
Rate of Exudation (ml./min. x 10⁻³)
24
CO2
Air
12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N. 12M. 12N.
Dec. 30-31 Jan. 1 2 3 4 5 6 7
Time (days)

FIG. 9. Termination of cycles of large amplitude following introduction of carbon dioxide for seven hours, and the effect of subsequent replacement of carbon dioxide by air at times indicated by arrows.

and negative exudation are 12 hours out of phase indicates that diurnal cycles in root permeability, by affecting the rate of movement of water within the root, cannot be responsible for the cycles observed in exudation unless unidirectional permeability is involved. However, the possibility remains that fluctuations either in the permeability of the absorbing root cells or in the energy available for respiration, by controlling the rate of water absorption from the soil, may be responsible for the cyclic nature of both positive and negative exudation.

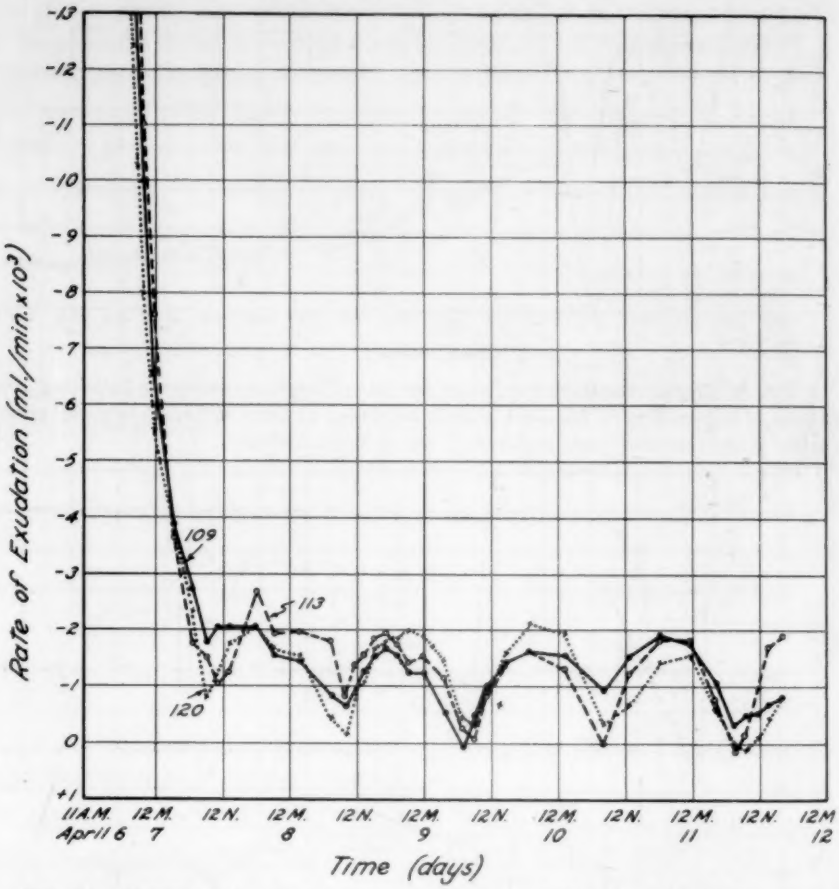

FIG. 10. Diurnal cycles in the rate of negative exudation by roots washed free of soil prior to the wilting and detopping of the plant.

To investigate the possible dependence of exudation periodicity on the transfer of water between roots and soil, negative exudation experiments were carried out on root systems washed free of soil and replaced into sealed cans containing saturated air. After the soil was removed and the roots replaced in the cans, the intact plant was allowed to wilt and was then detopped. Most of these bare root systems also displayed cycles

which followed the same pattern as those observed with roots in soil (see figures 10 and 11). The smaller amplitude in the cycles shown by the washed roots may have resulted from injury to a number of root tips incurred in washing the soil from the roots. The periodic behavior shown by the washed roots suggests that diurnal cycles in exudation are not dependent upon a periodic change in the rate of transfer of water between the soil and the roots. However, it is possible that during these experiments some water was excreted to the washed root surfaces and later reabsorbed.

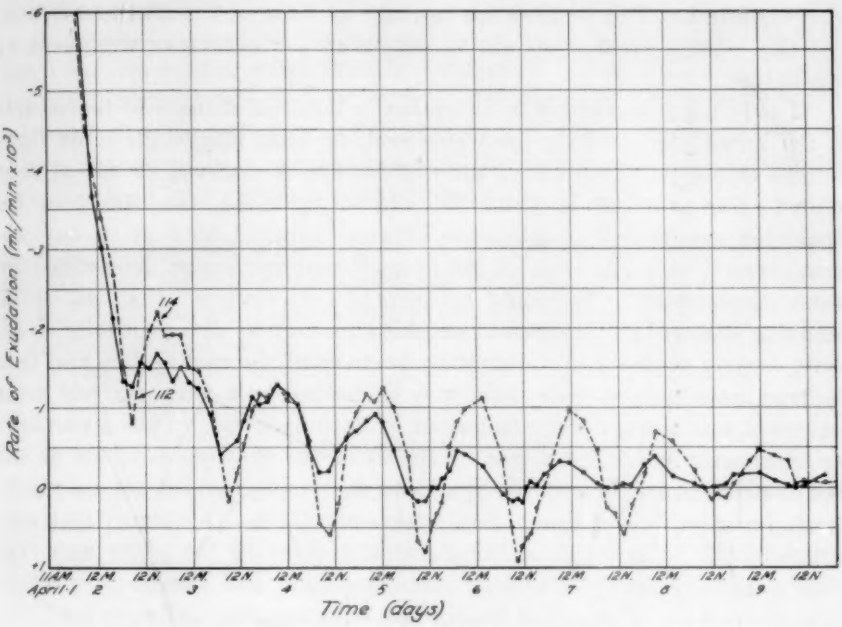

FIG. 11. Diurnal cycles in the rate of negative exudation by roots washed free of soil prior to the wilting and detopping of the plant.

GROSSENBACHER (**6**) offers the suggestion that cycles in positive exudation may arise as a result of cycles in root growth which he considers might regulate in a cyclic manner the rate of water absorption from the soil. While these experiments with washed roots cast doubt on the suggested dependence of exudation periodicity on a growth-controlled cyclic rate of water absorption from the soil, the possibility remains that cycles in root growth may contribute to the cyclic nature of the negative exudation observed in these bare roots. Further work on this point is needed.

A diurnal change in the hydration of the roots may be involved. ROSENE (**12**) has made simultaneous measurements of water absorption and exudation by onion root segments during 2-hour intervals. She finds that the ratio of the volume of exudate to the volume of water absorbed may depart appreciably from unity. Since her experiments were con-

tinued over a period of only 24 hours, it is not possible to detect the presence of a diurnal cycle. However, her observations would appear to indicate that the hydration of root segments may undergo appreciable change. In the writer's experiments some roots, for example No. 114 of figure 11, show an alternate positive and negative exudation. Since the periods of minimum positive exudation occur near noon and the periods of maximum negative exudation occur close to midnight, it appears that the hydration of the root system decreases as noon is approached and then increases to a maximum again near midnight. The importance of a varying hydration of cytoplasm has been pointed out recently by CRAFTS, CURRIER and STOCKING (**2**). Their treatise should be consulted for a further treatment of this subject.

If one considers changes in hydration in terms of changes in the volume of the liquid phase within the whole root, of what magnitude must these volume changes be to produce the diurnal cycles observed in the present study? For example, in plant No. 114 of figure 11, the difference between the maximum and minimum effective volumes on a given day approximates ½ to 1 per cent. of the over-all root volume as determined by water displacement. It should be pointed out, however, that the cyclic behavior observed in the present experiments may be due primarily to activity largely confined to the growing portions of the root system and that the true magnitude of these cycles may be masked by a comparatively large volume of root tissue not characterized by such behavior. Thus a periodicity of greater amplitude might be found in the growing portions of the root if measurements on this tissue alone were to be carried out over sufficiently long periods of time. Some years ago BOSE (**1**) claimed that pulsations could be detected in the growing regions of the plant and that these pulsations consisted of alternate expansions and contractions which were stopped by poisons, but these ideas have received little support.

It may be concluded from the experiments herein reported that the living roots of decapitated plants exhibit autonomic diurnal cycles in both positive and negative exudations which are dependent upon continued aeration. As yet no satisfactory explanation for this cyclic behavior can be offered.

Summary

1. Intake of water through the stumps of detopped root systems under a water deficit was investigated using field sunflower (*Helianthus annuus*) plants grown under the normal day-night environment of the greenhouse. Twelve-week-old plants were allowed to wilt, detopped under water, and then connected to a water supply in measuring pipettes. Water intake by cut stumps, termed negative exudation, proceeded at a high rate initially, approaching zero within a few days.

2. In these experiments conducted in a constant environment room, the rate of negative exudation by detopped root systems followed an auto-

nomic diurnal cycle with distinct maxima at midnight and minima at noon. This is in harmony with observations previously confined to roots showing positive exudation.

3. These diurnal cycles continued with decreasing amplitude for about six days. Replacement of the atmosphere around the roots with nitrogen or carbon dioxide promptly terminated the cycles.

4. Similar cycles were observed in roots washed free of soil prior to wilting and detopping. This indicated that the cause of the diurnal cycles lies within the root and is not dependent on a periodic transfer of water between root and soil.

5. In some cases, after the internal water deficit in the roots was satisfied, the roots exuded water from the stumps into the supply pipettes during the daytime hours and withdrew water at night. This suggests the existence of a diurnal cycle in the hydration of the cytoplasm of the root system equivalent to a volume change in the moisture content of roots approximating $\frac{1}{2}$ to 1 per cent. of the over-all root volume.

Acknowledgement is made to Dr. F. J. Veihmeyer and Dr. H. B. Currier for their continued encouragement and helpful criticisms and to Mr. D. W. Henderson who collaborated in the early phases of the work.

DIVISION OF IRRIGATION
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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THE INFLUENCE OF ROOTSTOCK ON THE MINERAL COMPOSITION OF VALENCIA ORANGE LEAVES

PAUL F. SMITH, WALTER REUTHER, AND ALSTON W. SPECHT

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Rootstocks have been shown (**7, 8, 9, 11, 13**) to exert an influence on tree growth, disease resistance, and fruit quality factors of various types of citrus. Recent work in California (**5, 6**) in which the leaves of orange, grapefruit, and lemon trees on a large number of different rootstocks were analyzed for several nutritive elements, strongly suggests that the amounts of these minerals in the scion leaves may be affected by the rootstock used.

Since one of the objectives of the nutritional research program at this station is an attempt to associate the mineral composition of citrus leaves with tree performance, fruit quality, and fertilizer utilization, it is desirable to know as much as possible about the effect of rootstock on the mineral content of the leaves. Some information on eleven nutrient elements has been obtained with Valencia orange trees on six different rootstocks, under Florida conditions, and is presented herewith.

Materials and methods

A rootstock test with Valencia orange was laid out in a randomized block arrangement in 1942 by other members of this station staff for the purpose of comparing the six rootstocks in respect to their effect on various aspects of tree growth, yield, and fruit quality. The rootstocks used were sour orange (*Citrus Aurantium*), Rough lemon (*C. Limon*), Rusk citrange (*Poncirus trifoliata* × *C. sinensis*), Bowen grapefruit (*C. paradisi*), Cleopatra mandarin (*C. reticulata*) and Parson Brown (sweet) orange (*C. sinensis*). The budded trees were two years old when the experiment started, and had been in the field five years when leaves were taken for analysis in late July, 1947. The soil is Lakeland fine sand located near Tavares, Florida. A complete mixed fertilizer (N, P, K, Mg, Mn, Zn, Cu, and B) was applied uniformly to all plots three times each year. During the first three years applications were made manually, while a mechanical distributor was used for the last two years. Dolomitic limestone was applied uniformly to the entire experimental area at intervals in amounts sufficient to maintain the acidity of the soil at about pH 5.5. No nutritional sprays were applied at any time.

Thirty-six 6-months-old leaves from non-fruiting shoots of the spring flush were collected in a uniform manner from each plot of three trees, with nine replications for each rootstock. The leaves were throughly scrubbed in a neutral detergent solution (sodium lauryl sulphate), rinsed four times in distilled water, dried to constant weight at 50° C, ground to a fine powder in a semi-micro Wiley mill provided with a nickel-coated

screen, and re-dried at 50° C just prior to analysis. This procedure was found to neither increase nor diminish the internal sodium or potassium content of citrus leaves.

The total amounts of nitrogen and phosphorus in the leaves were determined by adaptation of the colorimetric methods described by COTTON (**2**). Potassium, calcium, and magnesium were determined by turbidimetric methods adapted from the procedures described by PEECH and ENGLISH (**10**). Zinc was determined by the A. O. A. C. method (**1**), while manganese, copper, boron, iron, and sodium were determined spectrographically (**3**).

Results and discussion

It is not the purpose of this report to give the detailed results of the growth measurements made thus far on these trees. The experiment has not run long enough to permit final conclusions as to the relative merit or advantage of one rootstock over another. It is pertinent to know, however, that the trees were not equally vigorous on the different rootstocks during the first five years in the field. An indication of the relative sizes of the trees is shown by the mean trunk circumference measurements presented in table I. Also, the trees on certain of the rootstocks set fruit a year or two earlier than others, and this may influence, in some way, the mineral content of the leaves (table I). The trees on all rootstocks bore some fruit in 1947, but much heavier crops were set by the trees on Rough lemon and Rusk stocks.

The mean dry weight of the leaves was influenced significantly by the rootstock, yet there was no consistent relationship of leaf size with trunk circumference. The most vigorous trees, produced by Rough lemon stock, had the largest leaves. These were 16.7 per cent. larger than the smallest leaves which were found on the grapefruit stock. Although the total number of leaves per plant was not determined, it seemed apparent that they would vary in the same manner as the size of the trunk.

The percentage of ash in the leaves was less influenced by rootstock than was the dry weight. The leaves from the grapefruit rootstock plots showed the greatest ash content which seemed to be a reflection of the high potassium content induced by that stock.

With the exception of sodium, all of the chemical elements determined show significant differences in concentration due to rootstock. It is evident, therefore, that root systems from different genera of citrus exercise some selectivity in their nutrient uptake. This is based on the assumption that the leaf composition reflects the relative absorption of minerals by the roots. Leaves from the trees on grapefruit rootstock show high potassium content and this is gained at the expense of calcium and magnesium. No single element shows a perfect relation between the concentration in the leaf and the size of the tree, yet the two rootstocks that produced the largest trees (Rough lemon and Rusk citrange) also tended to induce the

TABLE I

SUMMARY OF TRUNK CIRCUMFERENCE, LEAF WEIGHT, ASH, AND CHEMICAL ELEMENT CONTENT OF FOLIAGE OF 5-YEAR-OLD VALENCIA ORANGE TREES ON SIX DIFFERENT ROOTSTOCKS

Rootstock	Trunk circ. (*mm.*)	Mean dry wt. per leaf (*mgm.*)	Per cent. in dry leaves						P.p.m. in dry leaves					
			Ash	N	P	K	Ca	Mg	Mn	Cu	Fe	B	Zn	Na
S.O.	165	267	10.49	2.55	0.136	2.11	2.26	0.488	69.0	4.97	74.7	67.3	16.7	485
G.F.	210	264	11.23	2.56	0.141	2.62	2.11	0.369	62.3	5.62	59.3	93.6	21.7	514
Cleo.	212	292	10.60	2.50	0.140	1.97	2.33	0.482	87.7	5.30	87.3	81.1	33.7	489
Sw.	239	282	10.58	2.63	0.150	2.30	2.16	0.408	53.3	7.28	81.7	84.1	18.7	515
Rusk	245	272	10.44	2.81	0.145	1.76	2.41	0.514	80.2	8.34	125.9	94.6	19.7	527
R.L.	288	308	10.89	2.74	0.141	1.98	2.44	0.510	85.0	5.32	101.6	79.7	20.3	497
Maximum diff. (Per cent.)	74.5	16.7	7.6	12.4	10.3	30.7	15.6	39.3	64.5	67.8	111.9	40.6	101.8	8.7
Sig. of treat.	†	†	†	†	*	†	†	†	†	†	†	†	†	.....
L.D. @ 5%	27	20	.38	0.13	0.010	0.22	0.20	0.049	14.7	2.24	13.3	13.1	4.8	.....
L.D. @ 1%	36	26	.51	0.17	.....	0.30	0.26	0.066	19.6	2.99	17.8	17.5	6.8	.....

S.O. = Sour orange; G.F. = grapefruit; Cleo. = Cleopatra mandarin; Sw. = sweet orange (Parson Brown); Rusk = Rusk citrange; R.L. = Rough lemon.

* Signifies that the odds for significance exceed 19: 1, but less than 99: 1.

† Signifies that the odds for significance exceed 99: 1.

L.D. Signifies the least difference for significance between any two means.

greatest concentration of many of the individual elements in the leaves. This is more readily seen in table II, where the rootstocks are ranked in accordance with the increase of each element as percentage of the leaf dry matter. The stock occurring in the upper position in each column contained the least amount of that particular element. The least vigorous trees, which were produced by sour orange and grapefruit stocks, tend to fall in the upper positions in the columns.

When the chemical constituents were expressed on an absolute basis (*i.e.*, mgm. per leaf) nitrogen alone showed a positive correlation with tree size. The correlation coefficient between the amount of nitrogen per leaf and the trunk circumference was 0.567 with 0.325 being required for 1 per cent. significance. These values were 6.8, 6.8, 7.3, 7.4, 7.6, and 8.4 mgm. N per leaf for sour orange, grapefruit, Cleopatra mandarin, sweet orange, Rusk citrange, and Rough lemon, respectively. None of the other elements showed a consistent trend in this respect. These differences in the absolute amounts of nitrogen would, in all probability, be magnified considerably if the total number of leaves per plant were to be taken into consideration. This suggests that, if the accumulation in the leaves of any one of the elements measured was a major factor in the different growth rates found, it was nitrogen.

Another point clearly shown by these data is that the concentrations of the micro-nutrient elements were influenced to a greater extent by rootstock than were those of the macro-nutrient elements. Thus, nitrogen, phosphorus, and calcium showed only about a 10 to 15 per cent. increase from the stock with the lowest leaf content to that with the highest. Iron and zinc, however, showed over 100 per cent. difference between the extremes, and manganese and copper showed about 65 per cent. each. This apparent differential capacity of rootstocks to forage for certain of the micro-nutrients may be a factor determining the adaptability of tree fruit stocks to various soil types.

The influence of rootstock on boron accumulation in the leaves closely agrees with the results of Roy (**12**) in which the same scion-stock combinations were compared in young nursery trees. Haas (**4**) found that the boron accumulation of the leaves of lemon, grapefruit, and Washington Navel orange was affected by certain rootstocks and, significantly that the order of arrangement was essentially the same as that found in the leaves of seedlings of the various rootstock varieties. The present findings agree with those of Haas (**4**) that sour orange rootstock results in a lower boron content of the leaves than does sweet orange stock.

Experience has shown that Rough lemon is better adapted to the light, sandy, ridge soils of Florida than other commonly used rootstocks. Young trees grow more rapidly and bear more heavily for a number of years when budded onto this stock. The present findings seem to suggest that the ability of Rough lemon roots to provide the tops of the trees with larger

TABLE II

RELATIVE RANKING OF ROOTSTOCKS IN RELATION TO THE MINERAL CONTENT OF VALENCIA ORANGE LEAVES WHEN EXPRESSED AS PERCENTAGE OF DRY MATTER

RANK*	TRUNK CIRC.	LEAF WT.	ASH	N	P	K	CA	MG	MN	CU	FE	B	ZN
1	*S.O.*	G.F.	Rusk	Cleo.	S.O.	Rusk	G.F.	G.F.	Sw.	S.O.	G.F.	S.O.	S.O.
2	G.F.	S.O.	S.O.	S.O.	Cleo.	Cleo.	Sw.	*Sw.*	G.F.	Cleo.	*S.O.*	*R.L.*	Sw.
3	Cleo.	Rusk	Sw.	*G.F.*	G.F.	*R.L.*	*S.O.*	Cleo.	*S.O.*	R.L.	Sw.	Cleo.	Rusk
4	Sw.	*Sw.*	*Cleo.*	Sw.	*R.L.*	S.O.	Cleo.	S.O.	Rusk	*G.F.*	Cleo.	Sw.	*R.L.*
5	Rusk	Cleo.	R.L.	R.L.	Rusk	Sw.	Rusk	R.L.	R.L.	Sw.	R.L.	G.F.	G.F.
6	R.L.	R.L.	G.F.	Rusk	Sw.	G.F.	R.L.	Rusk	Cleo.	Rusk	Rusk	Rusk	Cleo.

S.O. = Sour orange; G.F. = grapefruit; Cleo. = Cleopatra mandarin; Sw. = sweet orange (Parson Brown); Rusk = Rusk citrange; R.L. = Rough lemon.

* The rootstock in the uppermost position in each column represents the scion-rootstock combination with the smallest measurement for that particular factor, the other stocks being arranged in order of increasing magnitude down the columns. Stocks above the italicized ones do not differ significantly from each other, while all below differ significantly from the uppermost stock.

quantities of nutrients (especially nitrogen) than sour orange roots may account, at least in part, for the above observations. The larger leaf size resulting from the use of this stock may reflect a greater moisture-procuring capacity in light soil by the Rough lemon roots.

The possible effect of rootstock upon the mineral composition of the leaves of other tree crops, upon which leaf analysis is to be used as a diagnostic tool, should not be overlooked. The rootstocks used for citrus are essentially clonal, due to the predominance of nucellar seedlings produced by the seed of most of the species used for citrus stocks. Thus it is possible that the effect of stock on foliage composition is in sharper focus with citrus than with some other fruits, because of the clonal character of the stocks and the wide difference in species used.

Summary

Valencia orange leaves, from a randomized plot experiment involving six different rootstocks, were analyzed for total ash content and eleven chemical elements.

The results show that the rootstock is of considerable importance in determining the pattern of mineral composition of scion leaves. Highly significant differences in the percentages of nitrogen, potassium, calcium, magnesium, manganese, copper, boron, zinc, and iron attributable to rootstock influence were found. The sodium content of the leaves was not significantly affected by rootstock.

Rootstocks induced larger variations in the concentrations of the micronutrient elements found in the leaves than they did in the case of the macro-nutrient elements.

The absolute amount of total nitrogen per leaf appeared to be correlated with tree size. None of the other elements determined showed such a consistent trend. This suggests that there is a differential ability of the stock to supply nitrogen to the scions and this in turn contributes toward producing the different growth rates observed.

Division of Fruit and Vegetable Crops and Diseases
Bureau of Plant Industry, Soils and Agricultural
Engineering, U. S. Department of Agriculture,
Orlando, Florida and Beltsville, Maryland

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THE EFFECTS OF SOME INHIBITORS OF PHOTOSYNTHESIS UPON THE PHOTOCHEMICAL REDUCTION OF A DYE BY ISOLATED CHLOROPLASTS

FERGUS D. H. MACDOWALL[1]

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Introduction

Physiological research directed upon the activity of isolated chloroplasts has been spurred by the belief that the photochemical activity which chloroplasts exhibit *in vitro* is part of photosynthesis. If this is so, then those components of the photosynthetic apparatus which are active in isolated chloroplasts can thus be brought into closer range for study. The object of the present work was to find how closely the dye-reducing system of isolated chloroplasts is related to photosynthesis, by comparing the response of the former to various poisons and narcotics whose action upon the latter is already known, and to investigate the nature of the chloroplast apparatus through its activity.

It is believed that the photochemical and enzymatic machinery of that part of the photosynthetic mechanism which brings about the oxidation of water and the liberation of oxygen can be removed from the living cell intact in the chloroplast structure in a form such that it will continue to function *in vitro* in the presence of externally supplied oxidants. It was the presence of such oxidants in aqueous leaf extracts which permitted the action of freed chloroplasts in Hill's initial experiments (**7**) wherein the evolution of oxygen was followed spectrophotometrically via the conversion of haemoglobin to oxyhaemoglobin. Such oxidants must also have been important in the original experiments by HABERLANDT (**6**) and EWART (**3**), and in those investigations by MOLISCH (**16, 17**) and INMAN (**13, 14**) which followed.

HILL (**8**) found that ferric potassium oxalate may be substituted for these unknown oxidants, and more recently WARBURG and LÜTTGENS (**20**) struck a close analogy to photosynthesis through the successful use of p-benzoquinone which accepts hydrogen in its reduction, unlike Fe^{+++} in the Hill reaction but like anabolites in photosynthesis. HOLT and FRENCH (**10**) found that chromate and ferricyanide can be reduced by the action of isolated chloroplasts with the evolution of oxygen, and developed a technique involving the visible reduction to their leuco- forms of several redox indicators (referred to here as "dyes"). The reduction of such dyes is now followed photometrically by the employment of a photronic cell, with appropriate filters, connected to a galvanometer. This method was used in the poisoning studies reported below and will be described in more detail in a forthcoming paper by HOLT, SMITH and FRENCH (**12**).

[1] Present address: Carnegie Institution of Washington, Division of Plant Biology, Stanford, California.

The following brief summary of evidence gleaned from past work on isolated chloroplasts is included here in support of the belief that the activity of isolated chloroplasts is homologous with a basic part of the photosynthetic mechanism:

1) Light is necessary; therefore, photochemical reactions are involved.

2) The quantum yield of the Hill reaction (FRENCH and RABIDEAU (**4**)) sometimes approaches the efficiency of photosynthesis in Chlorella.

3) Photochemical dye reduction occurs efficiently in red light which the dye (phenol indophenol at pH 6.5) does not absorb, so that at least certain chloroplast pigments do the sensitizing as in photosynthesis. Hill found that the most active wave lengths for his reaction are those around 600 mμ, which points to the same conclusion.

4) Etiolated chloroplasts of Swiss chard do not reduce dye under strong illumination of thirty minutes duration, which suggests the necessity of chlorophyll. Furthermore, J. H. C. Smith, C. S. French and V. Koski have recently found that the dye-reducing activity of chloroplasts from etiolated barley seedlings which were exposed to light of increasing duration, is directly proportional to the amount of chlorophyll resulting in the chloroplasts.

5) The light saturation which is characteristic of photosynthesis is also exhibited in the activity of isolated chloroplasts.

6) The reaction system of isolated chloroplasts includes one or more heat labile enzymatic reactions. Activity is completely lost in 15 minutes at 50° C.

7) The stoichiometry of the Hill reaction (wherein the evolution of oxygen is measured manometrically) and of the reactions in which quinone and the dye phenol indophenol are the oxidants respectively, shows that an oxide, like water in photosynthesis, is split by isolated chloroplasts as follows:

$$4\ Fe^{+++} + 2\ H_2O \longrightarrow 4\ Fe^{++} + 4\ H^+ + O_2$$
$$2\ Q + 2\ H_2O \longrightarrow 2\ QH_2 + O_2$$
$$2\ Ind + 2\ H_2O \longrightarrow 2\ IndH_2 + O_2$$

8) HOLT and FRENCH (**9**) found that the molar ratio of hydrogen ion (H^+) produced to oxygen (O_2) produced in the Hill reaction is 4:1 as in water ($2H_2O$).

9) In recent experiments with water with H_2O^{18}, HOLT and FRENCH (**11**) have shown that the oxygen evolved by the photochemical activity of isolated chloroplasts, in solutions of the various effective oxidants, comes from water.

10) In the absence of CO_2, intact cells of Chlorella evolve oxygen in light in the presence of Hill's solution (**10**) or p-benzoquinone (**21**) as do also Scenedesmus cells in solutions of the latter (**1**).

Further evidence for the belief that the photochemical activities of chloroplasts in the test tube and in the living cell are in fact homologous

rather than analogous phenomena is afforded here by the response of isolated chloroplasts to inhibitors of photosynthesis. Conversely, the effects of some inhibitors upon photosynthesis can be further characterized through studies on isolated chloroplasts. The fact must be borne in mind that only a part of the photosynthetic mechanism is isolated with the chloroplasts, as that part which is directly involved in the participation of carbon dioxide is inactivated in the process of isolation (with the death of the cell).

Methods

Isolation of chloroplasts

Because of the need for dependable supply and activity through the winter months, the chloroplasts used in these experiments were taken from the leaves of spinach (*Spinacia oleracea*) purchased fresh from the market, and of Swiss chard (*Beta vulgaris*) from the greenhouse. A small handful was first washed and the midribs removed. The chloroplasts were then freed by maceration for one minute in a cooled Waring Blendor with about 140 ml. of water. The mash was then strained through a cotton cloth and the resulting filtrate centrifuged at 800 g. in a small-angle centrifuge. The chloroplast portion of the precipitate was resuspended in a small volume of water (5 to 10 ml.). The whole operation was carried out as near to 0° C as possible, and resulted in a concentrated suspension of chloroplasts which was then stored in a test tube in an icebox close to 0° C.

Reaction mixtures and procedures

The reaction vessel was a rectangular glass cell of $1 \times 1 \times 5$ cm. inside dimensions. Three ml. of liquid in it could be fully illuminated by the light beam striking the vessel as placed in position before a photronic cell in a thermostat. As this volume was convenient to use, 1.5 ml. of the dye mixture (consisting of 10^{-4} M phenol indophenol, 0.08 M KCl, and 0.04 M phosphate buffer at pH 6.5) was run into the reaction vessel, and 1.5 ml. of the inhibitor solution to be tested—or distilled water in the case of the control—was added to it. A small volume of the chloroplast suspension (usually 0.04 to 0.1 ml. and containing between 0.03 and 0.08 mg. chlorophyll) was added to this mixture, and the chloroplasts were suspended homogeneously by gentle shaking. The chlorophyll content was found by extraction of samples with methyl alcohol and by determination with a Klett-Summerson photoelectric colorimeter.

The chloroplasts were left in the reaction mixture for five minutes in the dark at 20° C, the vessel being placed in a metal box held in the thermostat. This allowed sufficient time for the attainment of temperature equilibrium, and sufficient pretreatment for the poison or narcotic to render its effects almost complete. Readings were taken of the galvanometer deflection, which increased with the amount of light striking the pho-

tocell as the dye was reduced. The selected time for total dye reduction in the control reaction mixture during illumination ranged from two to six minutes, and readings were taken as frequently as eight times per minute when necessary.

The experiment designed to find the effects of an inhibitor consisted of a series of runs with conditions constant and with reaction mixtures identical except in the concentration of the inhibitor. Control runs were interposed between those with different concentrations of inhibitor, so that the loss of chloroplast activity with time could be determined.

In order to find whether an inhibitor affects a light (photochemical) or a dark (enzymatic) reaction, the rates of dye reduction by the control and poisoned mixtures were determined at a series of intensities of continuous illumination, obtained by using wire screens of known transmission, and the light-saturation curves were compared. When an inhibitor acts

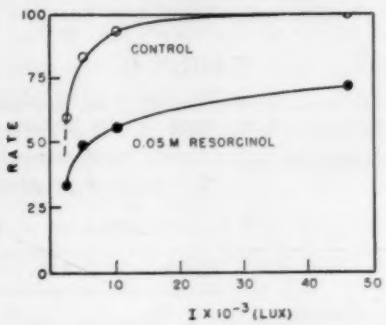

FIG. 1. Light-saturation curves showing the effect of 0.05 M resorcinol upon the rate of dye reduction by isolated chloroplasts at a series of intensities of continuous illumination.

upon a dark reaction which limits the rate at high light intensities, its effect is most evident at these high intensities (e.g. fig. 4). The inhibition of a photochemical reaction, on the other hand, shows up at low light intensities where light is limiting the rate of the overall reaction (e.g. fig. 1). These criteria have been used by workers in studies on photosynthesis.

Some physiologists have held that if an inhibitor of photosynthesis does not shift the absorption spectrum of chlorophyll in the leaf its effect is not due to an attack on chlorophyll. Some photochemists prefer to have further evidence, such as the absence of any influence upon the fluorescence of chlorophyll.

In the present work, however, tests were made only with absorption spectra, and runs were made with chloroplast suspensions and various separated pigments, as recorded in the results, in the presence of certain of the more important inhibitors. A Beckmann spectrophotometer was used for the determination of absorption spectra, and those obtained for chloroplast suspensions are surprisingly sharp.

Computations and errors

The maximum slope of the experimentally recorded curve describing the logarithm of the galvanometer deflection against time was taken as the rate of dye reduction. The galvanometer deflections were plotted logarithmically in order to obtain a linear relationship between dye concentration and time in accordance with Beer's law.

Some suspensions of chloroplasts lose their activity very rapidly after preparation. Although fresh Swiss chard chloroplasts were more active than those of spinach from the market, the former lost their activity much more rapidly—up to 50 per cent. in five hours. When necessary such an activity loss was corrected for in order to put all rates on the same basis. This was done by expressing the inhibited rate as per cent. of the control rate as taken from the curve for loss of activity with time.

The results obtained with a given sample of chloroplasts were generally reproducible within 10 per cent. However, because of some variation in

TABLE I

The dependence of the inhibitory action of two reagents upon the concentration of chlorophyll in the reaction mixture.

Inhibitor	% Inhibition at different molar concentrations of chlorophyll (contained in the chloroplasts) in the reaction vessel		
	3.6×10^{-5} M	1.8×10^{-5} M	3.6×10^{-6} M
2×10^{-5} M o-phenanthroline	38	41	43
2.5×10^{-3} M thymol	71	73	85

responses to inhibitors between different samples of chloroplasts, even from the same species of plant, it is not safe to state the results of this work in rigidly quantitative terms. As already mentioned the amount of chlorophyll contained in the chloroplasts used in the reaction vessel throughout this work varied with experiments between 0.03 and 0.08 mg., which is between 10^{-5} and 3×10^{-5} molar concentrations relative to the volume of fluid in the reaction vessel. At the most then, if the results obtained with o-phenanthroline and thymol (table I) can be generalized, such differences would not cause more than 5 per cent. variation in a measurement of inhibited activity. Nevertheless, caution should be observed in comparing the results of the present inhibition experiments with those on photosynthesis obtained by other workers who have not measured or recorded chlorophyll concentrations.

Results

In those figures below which describe the effects of inhibitors upon the rate of dye reduction by isolated chloroplasts, the molar concentration of the inhibiting reagent is plotted logarithmically on the abcissa against the per cent. of the control (unpoisoned) rate on the ordinate.

Poisons for metallo-catalysts

Cyanide—It was found that the rate of dye reduction by isolated chloroplasts is retarded 15 to 20 per cent. by 10^{-2} M KCN. Cyanide therefore does not poison this system specifically.

Azide—Azide also is not a specific poison for the present reaction system as 50 per cent. inhibition is brought about by 0.08 M sodium azide. Tests at two intensities of light with Swiss chard chloroplasts showed that the inhibition by 10^{-3} M azide was less at the lower intensity, thus indicating that a dark reaction is affected. The results were as follows:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	68%	83%

Pyrophosphate—This substance like cyanide is considered to be an inhibitor of certain iron-catalyzed reactions. Difficult solubility limited the concentration of the poison stock solution to 0.2 M, and it was necessary to use sodium pyrophosphate and sodium acid pyrophosphate in the appropriate proportions to give a poison solution of approximately pH 6.5.

The results, corrected for the loss of chloroplast activity with time, show that pyrophosphate up to 0.05 M concentration does not affect the rate of dye reduction by isolated chloroplasts.

Hydroxylamine—It was found here that a mean value, between samples of chloroplasts from spinach and Swiss chard leaves, of 3.1×10^{-4} M hydroxylamine hydrochloride caused 50 per cent. inhibition in the rate of dye reduction. The mean amount of chlorophyll in the chloroplasts used in the reaction vessel through these tests was 0.042 mg. which is a concentration of approximately 1.6×10^{-5} M. There was wider variation in the concentrations of poison causing complete inhibition and causing no inhibition between the different samples; for example, 9×10^{-4} to 4×10^{-3} in the former instance. Hydroxylamine can reduce the dye autonomously, but at concentrations of the poison which are critical for chloroplasts the rate of this reduction is not significant relative to the action of the chloroplasts. Results can readily be corrected for such a factor if necessary.

Somewhat irregular results were obtained in experiments on the effects of hydroxylamine at different intensities of light with either one of the dyes phenol indophenol or 2,6-dichlorophenol indophenol. For example, the following results were obtained with 10^{-3} M hydroxylamine:

Light intensity (lux):	32,400	15,900	7,650	3,800	1,800
Per cent. of control rate:	53%	48%	50%	44%	46%

However, the data all show that this poison retards the activity of chloroplasts by a constant fraction at the different intensities, and/or, because of a slight trend toward a greater effect at lower intensities, that it acts upon a light reaction.

It was found that hydroxylamine does not shift the absorption spectra of leaf xanthophylls in ether nor of chlorophyll *a* in ether, nor the red absorption maximum of chlorophyll in active chloroplasts (at poisonous concentrations). The interpretation of this evidence, which stands or falls on the validity of the spectrum-shift criterion, makes it unlikely that the effect of hydroxylamine is due to a direct combination of the poison with one of the above substances.

o-PHENANTHROLINE—Samples of chloroplasts containing a mean value of 0.034 mgm. chlorophyll (1.3×10^{-5} M) were completely inhibited by a mean concentration of 1.3×10^{-4} M o-phenanthroline (a 1:10 ratio of chlorophyll: o-phenanthroline), were retarded 50 per cent. by a mean concentration of 2.7×10^{-5} M o-phenanthroline, and were not affected by about 3.8×10^{-6} M o-phenanthroline at the highest.

The action of zinc sulphate in preventing the inhibition of the activity of isolated chloroplasts by o-phenanthroline, as shown by WARBURG and LÜTTGENS (**22**), was confirmed here. Tests were made at several concen-

TABLE II

THE PREVENTION, BY SOME METALS, OF THE INHIBITION OF DYE REDUCTION BY O-PHENANTHROLINE.

REAGENTS IN MIXTURE	PER CENT. OF CONTROL RATE OF DYE REDUCTION BY CHLOROPLASTS
10^{-4} M o-phenanthroline	37%
" + 4.7×10^{-5} M $CuSO_4$	115%
" " $CoSO_4$	104%
" " $NiSO_4$	92%
" " $FeSO_4(NH_4)_2SO_4$	66%

trations of $ZnSO_4$ such that it could be shown that approximately 2×10^{-5} M $ZnSO_4$ completely prevented the inhibition caused by 10^{-4} M o-phenanthroline upon a sample of spinach chloroplasts, whereas 3.8×10^{-6} M was the highest concentration of $ZnSO_4$ having no effect. Cupric sulphate, cobaltous sulphate, nickelous sulphate, and ferrous ammonium sulphate were also tried and found to prevent the inhibition by o-phenanthroline in that order (table II), but $ZnSO_4$ was the most effective. This prevention of inhibition is not the same thing as reversal.

The rate of dye reduction was measured for control and poisoned mixtures at a series of intensities of continuous light (table III). The results show that o-phenanthroline inhibits a light reaction. It was also found that this poison does not shift the red absorption maximum of chlorophyll in poisoned chloroplasts.

RESORCINOL—In the light of WARBURG and LÜTTGENS' (**22**) conclusion that o-phenanthroline inhibits by combining with catalytically active zinc, it was decided to try here another means of tying up zinc. Of the cations occurring in the chloroplasts only zinc should react with resorcinol, at least under analytical conditions. As shown in figure 2, it was found that

resorcinol inhibits dye reduction by isolated chloroplasts (from Swiss chard) only at high concentrations, so that unlike o-phenanthroline, it is not a specific poison. Whether or not resorcinol can combine with zinc in phosphate buffered media at pH 6.5 is not certain.

Trials at different light intensities (fig. 1) show that both light and dark reactions are inhibited by 0.05 M resorcinol.

THIOUREA—Dye reduction by Swiss chard chloroplasts containing 0.052 mg. chlorophyll was retarded 50 per cent. by 0.15 M thiourea. Inhibition started at about 10^{-2} M and was complete at about 2 M (by extrapolation). This poison can reduce the dye, but again this rate is negligible relative to the action of chloroplasts at effective concentrations of poison.

8-HYDROXYQUINOLINE—This substance is not very soluble in water

TABLE III

THE EFFECT OF O-PHENANTHROLINE UPON THE RATE OF DYE REDUCTION BY CHLOROPLASTS AT A SERIES OF LIGHT INTENSITIES.

CONCN. O-PHENANTHROLINE	% CONTROL RATE AT DIFFERENT LIGHT INTENSITIES (LUX)					
	46,300		10,200	4,900		2,100
	1	2	1	1	2	1
Samples 1, 2: 10^{-4} M	22%	18%	17%	12%	17%	10%
	6,700		4,400	2,200		1,000
Sample 3*: 10^{-5} M	70%		60%	48%		47%

* 2,6-dichlorophenol indophenol used as the dye (redox indicator) with different apparatus and at 15° C.

and gives a yellow solution. However, it did not reduce the dye and was apparently not affected itself by the activity of the chloroplasts. Because of solubility difficulties it was not used above 2.2×10^{-3} M in the reaction mixture. This concentration gave 22 per cent. inhibition. It can combine with many metals including zinc.

HEAVY METALS

COPPER SULPHATE—The rate of dye reduction by isolated Swiss chard chloroplasts containing 0.066 mg. chlorophyll (2.5×10^{-5} M) was retarded 50 per cent. by approximately 10^{-5} M $CuSO_4$. The results of rate measurements at a series of intensities of light show, as follows, that $CuSO_4$ (10^{-4} M) poisons a light reaction:

Light intensity (lux):	46,300	10,200	4,900	2,200
Per cent. of control rate:	39%	29%	16%	7%

The results obtained by GREENFIELD (5) with $CuSO_4$ and the photosyn-

Computations and errors

The maximum slope of the experimentally recorded curve describing the logarithm of the galvanometer deflection against time was taken as the rate of dye reduction. The galvanometer deflections were plotted logarithmically in order to obtain a linear relationship between dye concentration and time in accordance with Beer's law.

Some suspensions of chloroplasts lose their activity very rapidly after preparation. Although fresh Swiss chard chloroplasts were more active than those of spinach from the market, the former lost their activity much more rapidly—up to 50 per cent. in five hours. When necessary such an activity loss was corrected for in order to put all rates on the same basis. This was done by expressing the inhibited rate as per cent. of the control rate as taken from the curve for loss of activity with time.

The results obtained with a given sample of chloroplasts were generally reproducible within 10 per cent. However, because of some variation in

TABLE I

The dependence of the inhibitory action of two reagents upon the concentration of chlorophyll in the reaction mixture.

Inhibitor	% Inhibition at different molar concentrations of chlorophyll (contained in the chloroplasts) in the reaction vessel		
	3.6×10^{-5} M	1.8×10^{-5} M	3.6×10^{-6} M
2×10^{-5} M o-phenanthroline	38	41	43
2.5×10^{-3} M thymol	71	73	85

responses to inhibitors between different samples of chloroplasts, even from the same species of plant, it is not safe to state the results of this work in rigidly quantitative terms. As already mentioned the amount of chlorophyll contained in the chloroplasts used in the reaction vessel throughout this work varied with experiments between 0.03 and 0.08 mg., which is between 10^{-5} and 3×10^{-5} molar concentrations relative to the volume of fluid in the reaction vessel. At the most then, if the results obtained with o-phenanthroline and thymol (table I) can be generalized, such differences would not cause more than 5 per cent. variation in a measurement of inhibited activity. Nevertheless, caution should be observed in comparing the results of the present inhibition experiments with those on photosynthesis obtained by other workers who have not measured or recorded chlorophyll concentrations.

Results

In those figures below which describe the effects of inhibitors upon the rate of dye reduction by isolated chloroplasts, the molar concentration of the inhibiting reagent is plotted logarithmically on the abcissa against the per cent. of the control (unpoisoned) rate on the ordinate.

Poisons for metallo-catalysts

Cyanide—It was found that the rate of dye reduction by isolated chloroplasts is retarded 15 to 20 per cent. by 10^{-2} M KCN. Cyanide therefore does not poison this system specifically.

Azide—Azide also is not a specific poison for the present reaction system as 50 per cent. inhibition is brought about by 0.08 M sodium azide. Tests at two intensities of light with Swiss chard chloroplasts showed that the inhibition by 10^{-3} M azide was less at the lower intensity, thus indicating that a dark reaction is affected. The results were as follows:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	68%	83%

Pyrophosphate—This substance like cyanide is considered to be an inhibitor of certain iron-catalyzed reactions. Difficult solubility limited the concentration of the poison stock solution to 0.2 M, and it was necessary to use sodium pyrophosphate and sodium acid pyrophosphate in the appropriate proportions to give a poison solution of approximately pH 6.5. The results, corrected for the loss of chloroplast activity with time, show that pyrophosphate up to 0.05 M concentration does not affect the rate of dye reduction by isolated chloroplasts.

Hydroxylamine—It was found here that a mean value, between samples of chloroplasts from spinach and Swiss chard leaves, of 3.1×10^{-4} M hydroxylamine hydrochloride caused 50 per cent. inhibition in the rate of dye reduction. The mean amount of chlorophyll in the chloroplasts used in the reaction vessel through these tests was 0.042 mg. which is a concentration of approximately 1.6×10^{-5} M. There was wider variation in the concentrations of poison causing complete inhibition and causing no inhibition between the different samples; for example, 9×10^{-4} to 4×10^{-3} in the former instance. Hydroxylamine can reduce the dye autonomously, but at concentrations of the poison which are critical for chloroplasts the rate of this reduction is not significant relative to the action of the chloroplasts. Results can readily be corrected for such a factor if necessary.

Somewhat irregular results were obtained in experiments on the effects of hydroxylamine at different intensities of light with either one of the dyes phenol indophenol or 2,6-dichlorophenol indophenol. For example, the following results were obtained with 10^{-3} M hydroxylamine:

Light intensity (lux):	32,400	15,900	7,650	3,800	1,800
Per cent. of control rate:	53%	48%	50%	44%	46%

However, the data all show that this poison retards the activity of chloroplasts by a constant fraction at the different intensities, and/or, because of a slight trend toward a greater effect at lower intensities, that it acts upon a light reaction.

It was found that hydroxylamine does not shift the absorption spectra of leaf xanthophylls in ether nor of chlorophyll *a* in ether, nor the red absorption maximum of chlorophyll in active chloroplasts (at poisonous concentrations). The interpretation of this evidence, which stands or falls on the validity of the spectrum-shift criterion, makes it unlikely that the effect of hydroxylamine is due to a direct combination of the poison with one of the above substances.

o-PHENANTHROLINE—Samples of chloroplasts containing a mean value of 0.034 mgm. chlorophyll (1.3×10^{-5} M) were completely inhibited by a mean concentration of 1.3×10^{-4} M o-phenanthroline (a 1:10 ratio of chlorophyll: o-phenanthroline), were retarded 50 per cent. by a mean concentration of 2.7×10^{-5} M o-phenanthroline, and were not affected by about 3.8×10^{-6} M o-phenanthroline at the highest.

The action of zinc sulphate in preventing the inhibition of the activity of isolated chloroplasts by o-phenanthroline, as shown by WARBURG and LÜTTGENS (**22**), was confirmed here. Tests were made at several concen-

TABLE II

THE PREVENTION, BY SOME METALS, OF THE INHIBITION OF DYE REDUCTION BY O-PHENANTHROLINE.

REAGENTS IN MIXTURE	PER CENT. OF CONTROL RATE OF DYE REDUCTION BY CHLOROPLASTS
10^{-4} M o-phenanthroline	37%
" + 4.7×10^{-5} M $CuSO_4$	115%
" " $CoSO_4$	104%
" " $NiSO_4$	92%
" " $FeSO_4(NH_4)_2SO_4$	66%

trations of $ZnSO_4$ such that it could be shown that approximately 2×10^{-5} M $ZnSO_4$ completely prevented the inhibition caused by 10^{-4} M o-phenanthroline upon a sample of spinach chloroplasts, whereas 3.8×10^{-6} M was the highest concentration of $ZnSO_4$ having no effect. Cupric sulphate, cobaltous sulphate, nickelous sulphate, and ferrous ammonium sulphate were also tried and found to prevent the inhibition by o-phenanthroline in that order (table II), but $ZnSO_4$ was the most effective. This prevention of inhibition is not the same thing as reversal.

The rate of dye reduction was measured for control and poisoned mixtures at a series of intensities of continuous light (table III). The results show that o-phenanthroline inhibits a light reaction. It was also found that this poison does not shift the red absorption maximum of chlorophyll in poisoned chloroplasts.

RESORCINOL—In the light of WARBURG and LÜTTGENS' (**22**) conclusion that o-phenanthroline inhibits by combining with catalytically active zinc, it was decided to try here another means of tying up zinc. Of the cations occurring in the chloroplasts only zinc should react with resorcinol, at least under analytical conditions. As shown in figure 2, it was found that

resorcinol inhibits dye reduction by isolated chloroplasts (from Swiss chard) only at high concentrations, so that unlike o-phenanthroline, it is not a specific poison. Whether or not resorcinol can combine with zinc in phosphate buffered media at pH 6.5 is not certain.

Trials at different light intensities (fig. 1) show that both light and dark reactions are inhibited by 0.05 M resorcinol.

THIOUREA—Dye reduction by Swiss chard chloroplasts containing 0.052 mg. chlorophyll was retarded 50 per cent. by 0.15 M thiourea. Inhibition started at about 10^{-2} M and was complete at about 2 M (by extrapolation). This poison can reduce the dye, but again this rate is negligible relative to the action of chloroplasts at effective concentrations of poison.

8-HYDROXYQUINOLINE—This substance is not very soluble in water

TABLE III

THE EFFECT OF O-PHENANTHROLINE UPON THE RATE OF DYE REDUCTION BY CHLOROPLASTS AT A SERIES OF LIGHT INTENSITIES.

CONCN. O-PHENANTHROLINE	% CONTROL RATE AT DIFFERENT LIGHT INTENSITIES (LUX)					
	46,300		10,200	4,900		2,100
	1	2	1	1	2	1
Samples 1, 2: 10^{-4} M	22%	18%	17%	12%	17%	10%
	6,700		4,400	2,200		1,000
Sample 3*: 10^{-5} M	70%		60%	48%		47%

* 2,6-dichlorophenol indophenol used as the dye (redox indicator) with different apparatus and at 15° C.

and gives a yellow solution. However, it did not reduce the dye and was apparently not affected itself by the activity of the chloroplasts. Because of solubility difficulties it was not used above 2.2×10^{-3} M in the reaction mixture. This concentration gave 22 per cent. inhibition. It can combine with many metals including zinc.

HEAVY METALS

COPPER SULPHATE—The rate of dye reduction by isolated Swiss chard chloroplasts containing 0.066 mg. chlorophyll (2.5×10^{-5} M) was retarded 50 per cent. by approximately 10^{-5} M $CuSO_4$. The results of rate measurements at a series of intensities of light show, as follows, that $CuSO_4$ (10^{-4} M) poisons a light reaction:

Light intensity (lux):	46,300	10,200	4,900	2,200
Per cent. of control rate:	39%	29%	16%	7%

The results obtained by GREENFIELD (5) with $CuSO_4$ and the photosyn-

thesis of Chlorella at a series of light intensities are shown for comparison as follows: (5×10^{-6} M $CuSO_4$ was used)

Light intensity (lux):	22,000	4,000	1,600	700	350
Per cent. of control rate:	54%	64%	72%	79%	69%

It was found that $CuSO_4$ does not shift the absorption spectrum of the chloroplast pigments in the chloroplast.

MERCURIC CHLORIDE—$HgCl_2$ poisoned the reaction mechanism of Swiss chard chloroplasts very specifically (fig. 2). For a concentration of chloro-

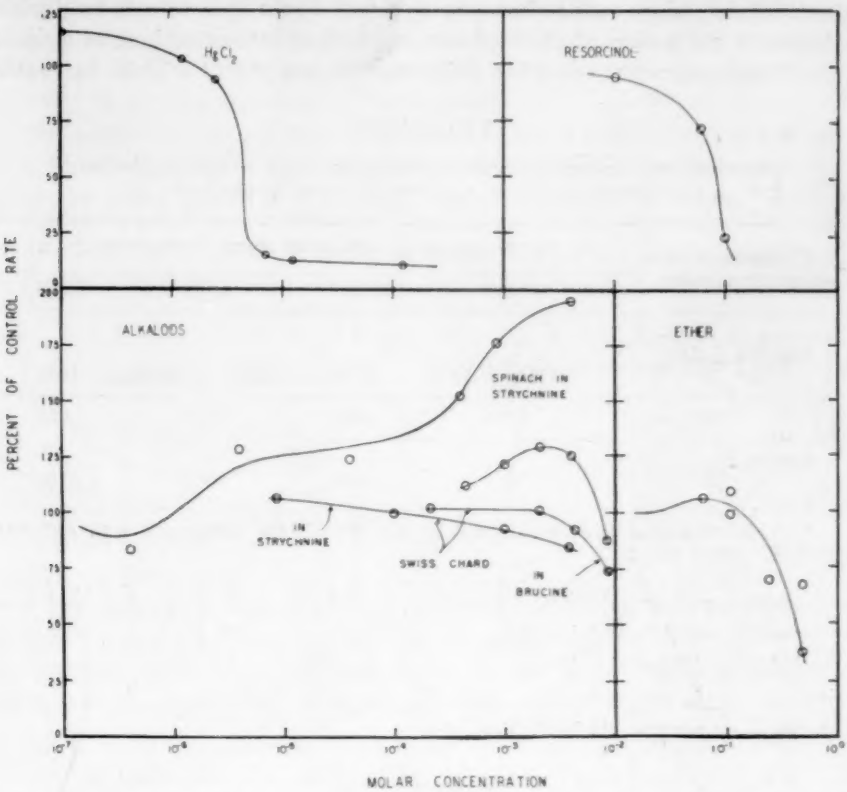

FIG. 2. The effects of a number of inhibitors upon the rate of dye reduction by isolated chloroplasts.

phyll in the reaction vessel of 7.4×10^{-5} M, concentrations of $HgCl_2$ below 10^{-6} M apparently stimulated the system, and 50 per cent. inhibition was brought about by 4×10^{-6} M poison. The absorption spectrum of the pigments in the chloroplast was not affected by even 10^{-3} M $HgCl_2$. Tests at different intensities of light gave the following results, showing that a dark reaction is inhibited by 6×10^{-6} M $HgCl_2$:

Light intensity (lux):	46,300	10,200	4,900	2,200
Per cent. of control rate:	14%	15%	17%	25%

OTHERS—Nickel, cobalt and zinc as their respective sulphate salts were tried without success because, within the concentration ranges in which they are known to affect photosynthesis, precipitation occurred in the presence of buffer (phosphate or borate) at pH 6.5.

OTHER SPECIFIC POISONS

DINITROPHENOL—Sodium, 2,4-dinitrophenol was found to have a specific effect upon the reduction of dye by isolated chloroplasts. Samples of chloroplasts from spinach and Swiss chard leaves, containing an average concentration of 1.3×10^{-5} M (0.035 mg.) chlorophyll, were inhibited 50 per cent. by a mean concentration of 6.3×10^{-4} M dinitrophenol. Variation between samples in concentrations of poison giving 50 per cent. inhibition was not great (4.5×10^{-4} M to 7.5×10^{-4} m), but incipient inhibition was brought about by concentrations ranging from 6×10^{-6} M to 10^{-4} M dinitrophenol, and complete inhibition occurred in solutions of 5×10^{-3} to 5×10^{-2} M poison.

Experiments showed that dinitrophenol inhibits more as the light intensity is decreased, i.e., that it inhibits a light reaction. For example, as follows with 10^{-3} M dinitrophenol:

Light intensity (lux):	6,700	4,350	2,200	1,000
Per cent. of control rate:	45%	37%	35%	28%

Because of the comparatively low light intensities used it is not certain whether or not the poison also affects a dark reaction.

This poison does not affect the spectral absorption maxima of pure chlorophyll *a* in ether solution, nor of the chlorophyll in poisoned chloroplasts. As was the case with most inhibitors tested in this work, the dye was not reduced by dinitrophenol.

IODOACETIC ACID—The dye-reducing activity of isolated Swiss chard chloroplasts was inhibited by iodoacetate over a wider inhibitory transition-range than was the photosynthesis of Chlorella as found by KOHN (**15**). The results from two of the present experiments are shown in figure 3. The rate of dye reduction was retarded 50 per cent. by approximately 10^{-2} M iodoacetic acid. Penetration of the poison was not the problem it was for live cells (Kohn), as a thirty-minute pretreatment to 10^{-2} M iodoacetate in the dark gave only 6 per cent. greater inhibition than did the regular five-minute pretreatment.

The following results show that a dark reaction is inhibited by 5×10^{-3} M iodoacetate, thus agreeing with Kohn's findings in photosynthesis:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	43%	56%

NARCOTICS

PHENYLURETHAN—The position of the inhibitory transition-range of phenylurethan in dye reduction by isolated spinach chloroplasts, fell at a

tenfold higher concentration range than that found by WARBURG (**20**) for the photosynthesis of Chlorella (fig. 3). In the present work, approximately 2×10^{-3} M phenylurethan brought about 50 per cent. inhibition, the concentration of chlorophyll within chloroplasts in the reaction vessel being 3.2×10^{-5} M (0.088 mg.).

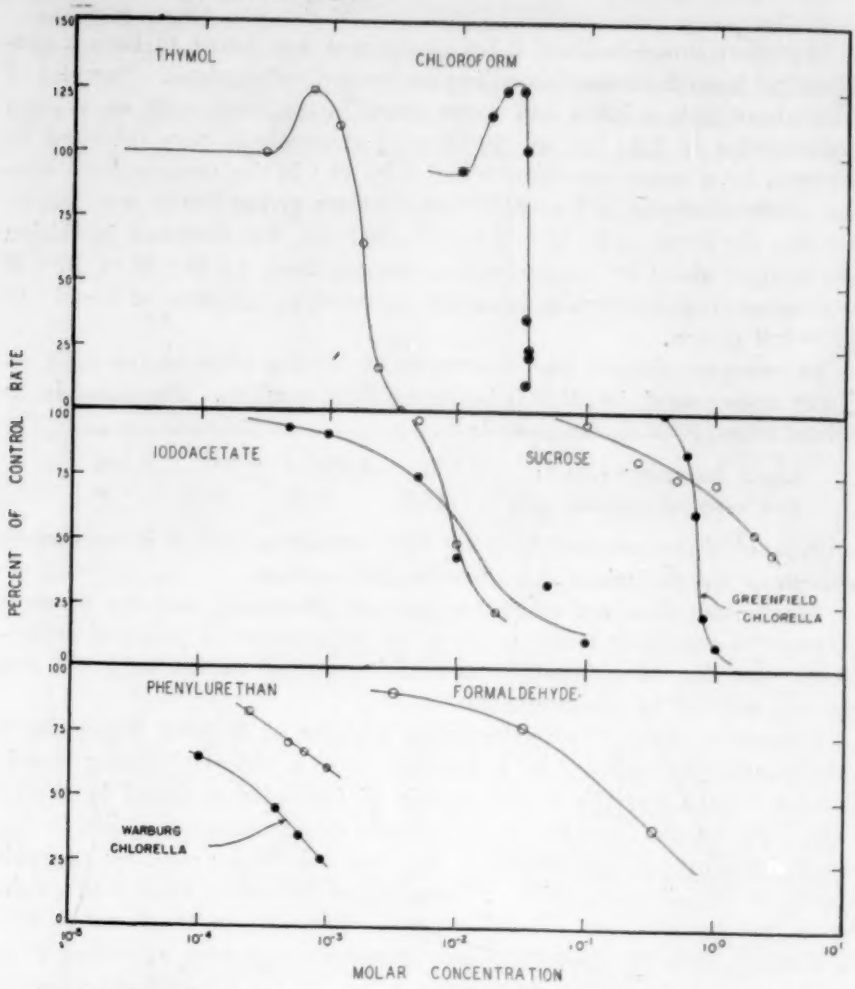

FIG. 3. The effects of a number of inhibitors upon the rate of dye reduction by isolated chloroplasts.

The stronger inhibition observed at the lower light intensity (table IV) means that phenylurethan inhibits a light reaction.

In accordance with the suggestion made by RABINOWITCH (**18**, p. 322), the absorption spectrum of active spinach chloroplasts (but *in vitro* here) was followed before and after treatment with strongly poisonous concentrations of phenylurethan. There was no shift in the absorption peaks of

chlorophyll in the chloroplasts so that the possibility of direct action of the narcotic upon chlorophyll is not favored by this evidence.

THYMOL—Thymol completely inhibited the photochemical activity of spinach chloroplasts at 2.6×10^{-3} M concentration and caused no change in activity at 1.2×10^{-3} M concentration (fig. 3). This sharpness of action might indicate a type of high specificity. The enhancing of chloroplast activity by concentrations of thymol immediately preceding those causing inhibition is of theoretical interest.

Tests run at series of intensities of continuous light showed that thymol

TABLE IV

THE EFFECTS OF SOME NARCOTICS UPON THE RATE OF DYE REDUCTION BY CHLOROPLASTS AT TWO LIGHT INTENSITIES.

INHIBITOR	CONCENTRATION	PER CENT. OF CONTROL RATE	
		46,300 LUX	4,900 LUX
Phenylurethan	5×10^{-4} M	71%	44%
	1×10^{-3} M	62%	47%
Chloroform	2.3×10^{-3} M	71%	57%
	2.6×10^{-2} M	43%	36%
Strychnine	4.8×10^{-3} M	194%	87%
	1.1×10^{-3} M	178%	78%
	4.8×10^{-4} M	153%	87%
	4.8×10^{-5} M	123%	80%

inhibits the rate of reaction equally well at all light intensities. The results of one experiment with 10^{-3} M thymol were as follows:

Light intensity (lux):	18,500	2,200	440
Per cent. of control rate:	51%	50%	52%

It was also found that thymol does not bring about any shift in the red absorption peaks of chromatographically separated chlorophylls *a* and *b* in ether. This evidence is contrary to that which might be expected if thymol combines with chlorophyll. Nor does this narcotic shift the red absorption peak of chlorophyll in active chloroplast fragments after their treatment by supersonic vibration. Hence, as is also the case with the other reagents which do not cause a shift in this spectrum, it also does not bring about a dissociation of the chlorophyll-protein complex. The absorption spectrum of pure carotene pigments in ether is not affected by thymol.

CHLOROFORM—The activity of spinach chloroplasts containing 0.065 mg. chlorophyll (2.4×10^{-5} M in the reaction vessel) was retarded 50 per cent. by 3.4×10^{-2} M chloroform and completely arrested by 3.5×10^{-2} M chloroform, which shows that this narcotic is about ten times less "specific" than thymol. The shape of the curve describing the inhibitory transition-range for chloroform (fig. 3) is very like that for thymol and shows a 25 per cent. stimulation of chloroplast activity at concentrations of the

narcotic just preceding those causing inhibition. Reversal was attempted by centrifuging and washing the chloroplasts followed by a second centrifuging and the resuspension of the chloroplasts in control reaction mixtures. Single treatments were partly successful (up to 50%).

According to the results obtained at two intensities of continuous illumination (table IV) chloroform inhibits a light reaction.

ETHER—The results obtained in the present experiments were fairly irregular, possibly because of the volatility of ether. To minimize this, ether solutions were made to desired concentration immediately in the reaction vessel and the chloroplasts were suspended by a single slow inversion of the vessel. The curve representing the inhibition by ether (fig. 2) is drawn as a mean curve through the values which were corrected for loss of chloroplast activity over the duration of the experiment. It can be seen that the inhibitory transition-range of ether is similar in form (with evidences of stimulation) to that of thymol and of chloroform, and that ether is the least specific narcotic. 0.45 M ether inhibited the rate of dye reduction of isolated Swiss chard chloroplasts by 50 per cent.

The rates of dye reduction in the presence of 3.5 per cent. ether (0.47 M) at two intensities of continuous illumination indicate that a dark reaction is inhibited by ether, as the rate is inhibited by 30 per cent. and 23 per cent. at light intensities of 20,600 and 2,200 lux respectively.

STRYCHNINE AND BRUCINE—The results obtained for the action of these alkaloids upon the activity of isolated chloroplasts, from several samples of leaves, are shown graphically in figure 2. Stimulation was again apparent for most samples. One sample of spinach chloroplasts showed a stimulation by 0.25 per cent. strychnine (3×10^{-3} M) which doubled its normal rate of dye reduction. Rates for this sample at two intensities of continuous illumination and with different concentrations of strychnine (table IV) show clearly that a dark reaction is stimulated by strychnine. One sample of Swiss chard (not in figure 4) was stimulated 17 per cent. by 0.17 per cent. strychnine (2×10^{-3} M).

The alkaloids, like the other narcotics and many poisons, did not reduce the dye autonomously.

OTHER INHIBITORS

SUCROSE—A study was made on the effect of the concentration of chemically inactive molecules, using solutions of sucrose of increasing concentration, upon the rate of dye reduction by isolated chloroplasts. The results obtained with a single sample of Swiss chard chloroplasts (fig. 3) show that the influence starts at a concentration of sucrose of about 0.08 M which corresponds to about 2.1 atmospheres of osmotic pressure, weight molar solutions having been used. GREENFIELD (**5**) studied the effect of dehydration by osmosis with sucrose solutions upon the rate of photosynthesis of Chlorella, and his results are reproduced in figure 3 as a matter of interest; however, they may not be directly comparable with the present results, as broken chloroplasts may not have semi-permeable surfaces.

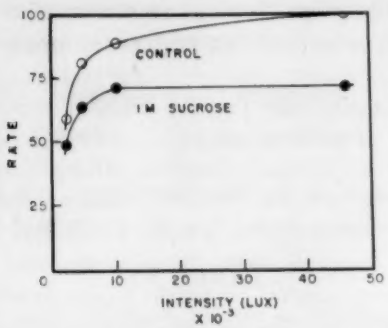

FIG. 4. Light-saturation curves showing the effect of 1 M sucrose upon the rate of dye reduction by isolated chloroplasts at a series of intensities of continuous illumination.

Curves describing the light saturation of the system (fig. 4) show that dark reactions are hindered by sucrose at a high concentration.

FORMALDEHYDE—BOSE (**2**) found that 10^{-7} per cent. formaldehyde increased the rate of photosynthesis of Hydrilla by 80 per cent.

The photochemical reduction of indophenol by freed chloroplasts of Swiss chard would be completely inhibited by nearly 10 per cent. (3.3 M) formaldehyde (fig. 3). No stimulation occurs down to 10^{-10} per cent.

TABLE V

THE EFFECTS OF OTHER SUBSTANCES UPON THE RATE OF DYE REDUCTION BY ISOLATED CHLOROPLASTS.

SUBSTANCE	CONCENTRATION	% OF CONTROL RATE
Methyl alcohol	5%	103
Ethyl alcohol	5%	91
	10%	39
	19%	17
Carbitol	5%	0
Isopropanol	5%	50
n-propyl alcohol	5%	50
Propylene glycol	5%	113
	10%	89
	20%	59
	30%	31
	40%	10
Acetone	5%	50
	10%	34
Diethylene glycol-monobutyl ether	5%	30
Glycerine	10%	80
Toluene	1%	70
Dioxane	10%	0
$MgSO_4$	2% sat'd.	25
	5% "	15
Na_2SO_4	1% "	96
	2% "	87
	4% "	70
	6% "	51
	10% "	43
$(NH_4)_2SO_4$	10% "	33
$CaCl_2$	0.2 M	0

(3×10^{-11} M) formaldehyde. Tests at two intensities of light showed that 0.3 M (1%) formaldehyde exercises its toxicity upon a light reaction:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	39%	20%

OTHERS—Previous work in this laboratory included some studies designed to find a stabilizing agent for the storage of isolated chloroplasts

TABLE VI

A SUMMARY OF THE EFFECTS OF VARIOUS INHIBITORS UPON THE PHOTOCHEMICAL REDUCTION OF INDOPHENOL BY ISOLATED CHLOROPLASTS.

INHIBITOR	MOLAR CONCN. GIVING 50% INHIBITION	MOLAR CONCN. RANGE GIVING 10–90% INHIBTN.	REACTION INHIBITED
Poisons for metallo-cats.:			
Cyanide	(>0.01)		
Azide	0.08	0.02 – 0.1	Dark
Pyrophosphate	(>0.1)		
Hydroxylamine	0.00031	0.00002 – 0.0006	(Light)
		0.0007 – 0.0014	Constant Fraction
o-Phenanthroline	0.000027	0.0000014 – 0.00001	Light
		0.000007 – 0.00013	
Resorcinol	0.09	0.012 – 0.1	Dark & Light
Thiourea	0.15	0.02 – 1.0	
8-Hydroxyquinoline	(0.08?)		
Heavy metals:			
Copper sulphate	0.00001		Light
Mercuric chloride	0.000004	0.000002 – 0.000005	Dark
Other specific poisons:			
Dinitrophenol	0.00063	0.00012 – 0.02	Light
		0.000015 – 0.0022	
Iodoacetate	0.01	0.003 – 0.03	Dark
Narcotics:			
Phenylurethane	0.002	0.00015 – 0.02	Light
Thymol	0.0019	0.0014 – 0.0029	Constant Fraction
Chloroform	0.034	0.034 – 0.035	Light
Ether	0.45	0.2 – 0.8	Dark
Strychnine	(>1.0)		Dark stimulated
Other inhibitors:			
Sucrose	2.3	0.12 – 5.0	Dark
Formaldehyde	0.18	0.02 – 1.2	Dark
Sodium sulphate	0.42		
Acetone	0.68		
Ethyl alcohol	1.36		

(**12**). The substances listed in table V were found by Holt and French to retard the rate of dye reduction as shown. These authors have kindly permitted the inclusion of this survey here. The visibly gauged time for decolorization of phenol indophenol was the measurement employed.

The effective concentrations of the various inhibiting reagents which have been tested in this work are summarized in table VI.

Discussion and summary

This paper reports a survey of the actions of some of the known inhibitors of photosynthesis upon the dye-reducing activity of chloroplasts freed *in vitro*. Related work has already been adequately reviewed (**18**). The dye used was phenol indophenol, and 2,6-dichlorophenol indophenol in several instances, and the chloroplasts were obtained from the leaves of spinach and Swiss chard. The object of the paper is to substantiate the belief that the photochemical activity of isolated chloroplasts (which includes the oxidation of water, the liberation of oxygen, and the reduction of added oxidants) is actually a part of photosynthesis; and to report on the basis of this fact the effects of various reagents with an eye toward the elucidation of the reaction system which operates in chloroplasts.

The specificity of the various inhibitors relative to one another may be judged from the summary of experimental results in table VI.

The following inhibitors act specifically upon isolated chloroplasts, as they do on photosynthesis: Mercuric chloride; copper sulphate; o-phenanthroline; hydroxylamine; dinitrophenol. The action of the narcotics is of course not specific in terms of concentration, but that on isolated chloroplasts parallels that on photosynthesis.

Among the poisons, cyanide, azide, pyrophosphate, thiourea, possibly 8-hydroxyquinoline, and iodoacetate, are not specific inhibitors of dye reduction by isolated chloroplasts. This indicates that neither iron nor copper is involved in the reaction system of isolated chloroplasts. The effects of these reagents upon photosynthesis are probably connected with reactions which are directly concerned with the participation of carbon dioxide in this process.

Contrary to the results obtained here (see the comparison in the preceding section) GREENFIELD (**5**) found that $CuSO_4$ primarily inhibits a dark reaction in the photosynthesis of Chlorella. It is therefore suggested that Greenfield's dark reaction is not involved in the dye-reducing mechanism of isolated chloroplasts but is concerned with the utilization of carbon dioxide in photosynthesis. In several ways the results with $HgCl_2$ contradict GREENFIELD'S (**5**) conclusion that this poison attacks and destroys chlorophyll. The fact that copper inhibits a photochemical reaction in isolated chloroplasts whereas mercury inhibits a dark reaction suggests either different specificities or a difference in degree of effect, toward or upon presumably sulfhydryl groups.

Mercuric chloride, iodoacetate, azide, resorcinol, ether and sucrose inhibit dark reactions (probably enzymatic). Strychnine stimulates a dark reaction, and the other narcotics also stimulate the system at concentrations which are just below those which inhibit. Copper sulphate, o-phenanthroline, dinitrophenol, phenylurethan, chloroform, resorcinol, formal-

dehyde, and possibly hydroxylamine inhibit a light (photochemical) reaction. Thymol inhibits equally well at all intensities of light, and it is safer to say the same for hydroxylamine, which indicates according to WELLER and FRANCK (**23**) that a light-activated enzyme is attacked.

SCHIAU and FRANCK (**19**) attributed the influence of phenylurethan upon the fluorescence of isolated chloroplasts to adsorption of the narcotic on the chlorophyll, thus suppressing the photochemical activity of the latter. It may be contradictory that this narcotic does not influence the absorption spectrum of chlorophyll in the chloroplast.

It is interesting to note that among the narcotics all three types of effect are found upon the usual response to light saturation, thus implying that despite the probable common action of these inhibitors in an "indiscriminate blocking of surfaces," each may nevertheless have specific effects in blocking certain surfaces more or less effectively.

The evidence obtained here shows that WARBURG and LÜTTGENS' postulated zinc enzyme, which is specifically poisoned by o-phenanthroline, is concerned in a photochemical reaction and is thus perhaps a chemical photocatalyst. The same is true of one or both of the hypothetical oxygen-liberating enzymes, which are specifically poisoned by hydroxylamine, and of the hydrogen transport agent which is specifically poisoned by dinitrophenol. Chlorophyll does not seem, by spectral analysis, to be affected by these poisons (and others). The simplest picture in alignment with known evidence and theory is that these three poisons concern themselves with only two catalysts, one or both of which are directly sensitized by chlorophyll. The evidence also indicates that there is at least one enzymatic dark reaction included in the reaction system of isolated chloroplasts, and that there are enzymatically active or essential sulfhydryl groups in the system.

It is a pleasure to express profound gratitude for his kindness and help throughout to Dr. French who recommended this project and in whose laboratories this work was carried out. It is also a pleasure to thank members of the Botany Department at the University of Minnesota and particularly members of the Division of Plant Biology of the Carnegie Institution of Washington at Stanford for their interest and for suggestions for the presentation of the data.

DEPARTMENT OF BOTANY
UNIVERSITY OF MINNESOTA
MINNEAPOLIS 14, MINNESOTA

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ORANGE FRUITING IN RELATION TO THE BLOSSOM OPENING PERIOD

A. R. C. HAAS

(WITH FIVE FIGURES)

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Introduction

The blossoming and fruit-setting of orange trees in southern California continues for a period of at least five weeks. Thus, in 1947 at the Citrus Experiment Station at Riverside, California, it was possible to make large collections of fully-open Washington navel orange blossoms from March 19 to April 22 inclusive. Less abundant blossom opening occurred for a week or two prior to and after these dates. In the case of Valencia orange trees, abundant blossom opening occurred from March 31 to May 6 inclusive, with some blossom opening prior to and after this period. Blossoms are usually produced in excessive numbers and blossom-thinning has often been suggested as a means of producing fewer fruits of larger size or fruits of better quality, or of preventing alternate bearing. The important question presents itself; namely, as to whether open orange blossoms have equal opportunities in the setting of fruit that remain until the picking stage and whether orange blossoms that open at different times contribute equally to fruit production. Production as well as quality is an important phase in citrus growing and production may involve fruit quality.

If an answer to the problem were based on the knowledge thus far available, it would be that the first blossoms that open possess the best opportunity to set fruits that could mature into the commercial crop. As the blossoming season advances a decrease occurs in the nitrogen and phosphorus content in the blossoms (**1, 2, 3**). The first blossoms to open therefore contain the most nitrogen and phosphorus. They also have early access to the other food supplies that are greatly reduced as the blossoming season progresses. Even though the above conclusion may appear to be a logical one, the experimental results show that just the opposite conclusion is correct and that other facts have to be taken into consideration.

Experimental trees

The orange trees used in this study were budded in 1916 on sweet orange as rootstocks and were located in the orchards at the Citrus Experiment Station in Riverside, California. The soil fertilizer treatment for each of the eight Washington navel orange trees was three-fourths pound of nitrogen from ammonium nitrate on February 2, 1947, and one pound of nitrogen from manure applied on September 25, 1947. The soil fertilizer treatment per tree square in the Valencia orange plot consisted of one and one-half pounds of nitrogen from ammonium nitrate (formerly am-

monium sulphate was used) in the spring and one and one-half pounds of nitrogen from manure, plus a covercrop in the fall. These Valencia orange trees were divided into two groups for the purpose of testing the effects of 2,4-D. Five trees were sprayed with 8 p.p.m. of 2,4-D on September 4, 1947, and five unsprayed trees served as controls, and the results are expressed accordingly. The zinc spraying and fumigation practices for all of the experimental trees were similar to those in common use in all of the orchards at the Experiment Station.

Tagging of orange blossoms

The opening of the orange blossoms was carefully observed in order to start the trials as soon as a sufficient number of blossoms were open. When more blossoms than the one open blossom occurred on a selected shoot, the freshly open one was tagged and the remaining blossoms, if any were present, were cut away in order to eliminate the varying competition between blossoms on a single shoot. On each date chosen for the tagging of freshly opened blossoms, a sample of such blossoms was also collected for chemical analysis.

In 1947, thirteen freshly opened blossoms were tagged on each of eight Washington navel orange trees (a total of 104 blossoms) on each of the following dates: March 19, 25, 31, April 8, 15, and 22 respectively. A total of 104 freshly opened blossoms (fourteen on the first tree and ten on the other trees) were tagged on the ten Valencia orange trees on each of the following dates: March 31, April 8, 15, 22, 29, and May 6 respectively. The 2,4-D-sprayed Valencia orange trees are represented by 64 blossoms tagged at each successive date whereas the non-sprayed trees are represented correspondingly by 40 blossoms.

Climatic factors

In March 1947, the mean maximum daily temperature was 71° F and the mean minimum daily temperature 44° F. For the last 16 days in March, these values were 72° and 47° F respectively. In April 1947 the mean maximum daily temperature was 77° F and the mean minimum daily temperature 47° F. However, from April 4 to 10 inclusive, while the mean maximum daily temperature was 76° F, the mean minimum daily temperature was 38° F (the minimum values ranging from 36° to 40° F during this period). This period experienced the most sustained minimum daily temperature during the entire blossoming season. In May the mean maximum daily temperature rose to 84° F and the mean minimum temperature to 52° F, whereas for the first 15 days of May the values were 87° and 52° F respectively. Thus, both mean daily temperatures increased during the blossoming season. Table I gives the maximum and minimum temperatures for each day in March, April, and May in 1947. The effect of such temperature changes on blossom opening and fruit production are difficult to evaluate without equipment for environmental control.

The rainfall (in inches) during the blossoming season was extremely light: March 21, .26; March 28, .11; April 4, .02; April 22, .05; and April 23, .04.

Blossom composition at various tagging dates

Figures 1 and 2 show that the lowest calcium and potassium content occurred in the open Washington navel and Valencia orange blossoms collected on April 8. The low minimum temperatures from April 4 to 10

TABLE I

MAXIMUM AND MINIMUM TEMPERATURES (°F) DURING THE 1947 ORANGE BLOSSOMING PERIOD

DAY	MARCH		APRIL		MAY	
	MAX.	MIN.	MAX.	MIN.	MAX.	MIN.
1	66	38	70	49	76	50
2	68	50	67	50	96	54
3	70	36	64	46	103	53
4	61	45	68	36	104	50
5	64	44	69	38	104	57
6	71	35	73	37	97	57
7	67	34	85	39	87	57
8	58	46	78	40	87	47
9	68	41	75	40	80	47
10	68	40	85	38	79	52
11	76	44	88	43	79	52
12	77	46	89	64	70	50
13	82	37	92	52	75	43
14	86	38	95	47	79	55
15	87	39	93	47	84	53
16	83	43	92	49	65	48
17	84	45	88	49	74	54
18	60	52	75	51	79	54
19	67	52	82	49	88	51
20	57	51	75	54	91	51
21	61	48	74	54	80	57
22	61	49	67	46	88	54
23	63	49	71	43	79	57
24	85	37	71	52	73	51
25	89	44	73	53	80	51
26	82	42	66	47	88	52
27	71	45	70	49	96	52
28	70	53	72	47	73	48
29	73	49	76	52	70	47
30	65	48	72	45	81	56
31	74	46	.....	.....	85	55
Mean	71	44	77	47	84	52

inclusive (36° to 40° F) may have had an effect on the absorption of these elements by the blossom. In both varieties the percentage of calcium in the dry matter of the blossoms rises after April 8. The potassium values also rise after April 8, only to fall again later probably as a result of a reduction in the reserve supply of potassium.

Washington navel orange fruit from tagged blossoms

The navel orange fruits were picked on January 6, 1948. The 104 open blossoms tagged on the successive dates in 1947 during the blossoming

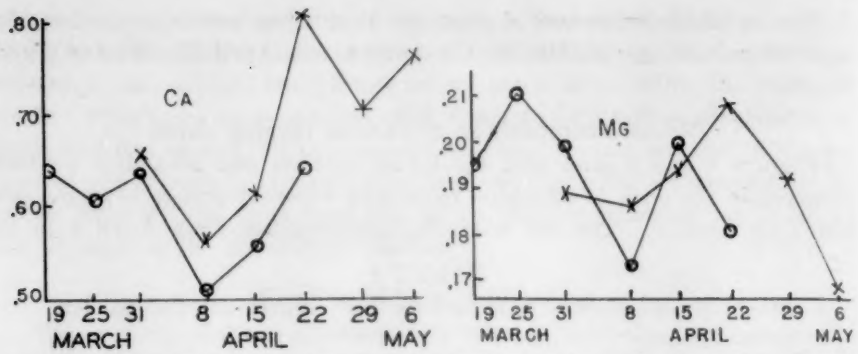

FIG. 1. Percentages of calcium and magnesium in the dry matter of open orange blossoms collected at various times during the blossoming season in 1947: 0, Washington navel orange; x, Valencia orange.

season resulted in the production of the following numbers of fruits of various sizes: March 19, 0; March 25, 7 fruits (3 of size 150 and 4 of size 200); March 31, 9 fruits (6—200's and 3—150's); April 8, 10 fruits (1—288, 1—244, 4—200's, and 4—150's); April 15, 17 fruits (2—288's, 9—200's, 5—150's, and 1—100); and April 22, 30 fruits (5—288's, 19—200's, and 6—150's) respectively.

A random sample of 103 fruits (1—344, 19—288's, 66—200's, and 17—150's) from non-tagged blossoms and picked from all parts of the trees on January 6, 1948, weighed 15,643 grams or 151.8 grams per fruit. The total fresh weight of fruits produced from the blossoms of the successive tagging dates were: 0, 1117, 1517, 1543, 2800, and 4900 grams respectively or 0, 159.6, 168.6, 154.3 (from the April 8, 1947 blossoms that opened during sustained low minimum temperatures), 164.7, and 163.3 grams respectively per fruit. Although the fruit samples in some cases were small, it appears that the fruit produced from the blossoms that opened during the cold period were the least heavy of those obtained from the tagged blossoms, and may indicate a relationship to the minimum temperatures.

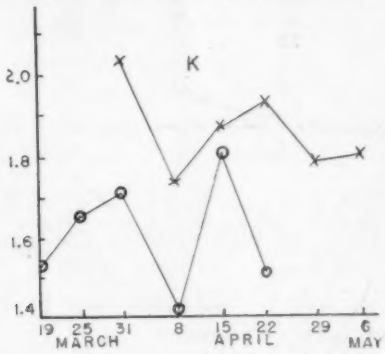

FIG. 2. Percentages of potassium in the dry matter of open orange blossoms collected at various times during the blossoming season in 1947: 0, Washington navel orange; x, Valencia orange.

Composition of Washington navel oranges

Though in some cases the fruit samples were small, yet here were fruits of similar size (200's was the size selected) and of different age, available for a study of variations in composition as a result of their different ages.

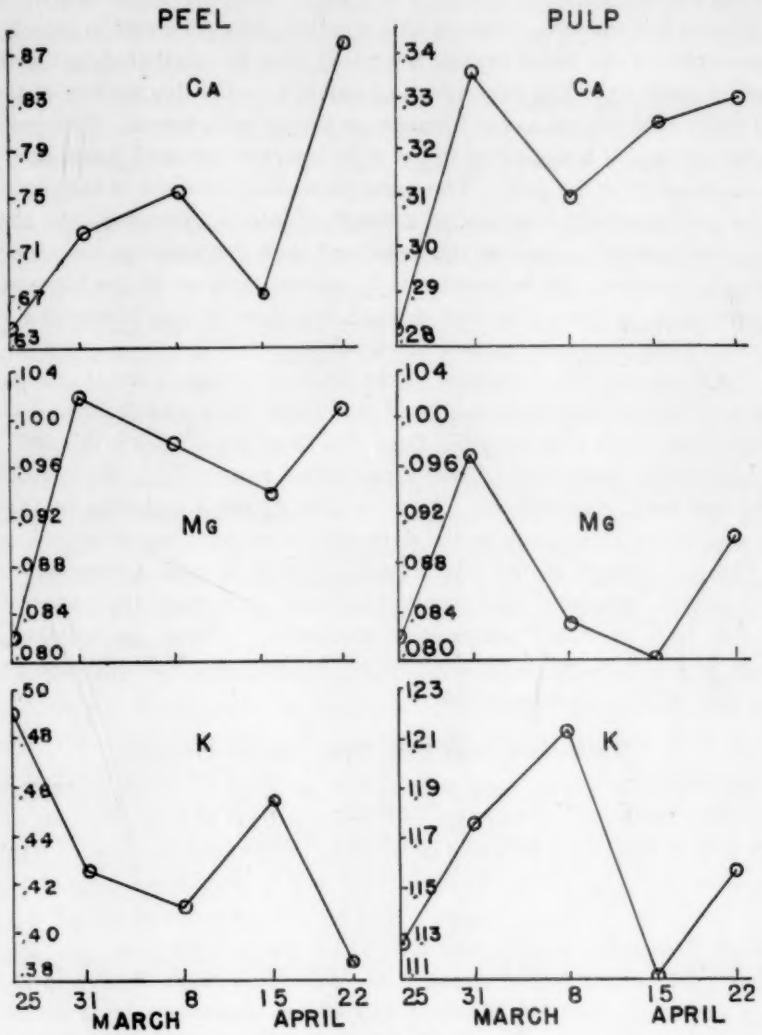

FIG. 3. Percentages of calcium, magnesium, and potassium in the dry matter of the peel and pulp of Washington navel oranges (size 200) produced from blossoms that opened on successive dates in 1947. The mature crop was picked on January 6, 1948.

The buttons were removed, the fruits were lightly brushed in running distilled water and wiped dry and weighed. The fruits were separated into peel and pulp [the secondary fruit (navel) was discarded] and the weights of the peel and pulp were obtained before they were dried at 65° C. As the

drying of separated pulp segments proceeded in the ventilated oven, the segments were torn further open to allow more rapid drying. Finally the peel was ground in a Wiley mill, whereas the pulp was pulverized in a porcelain mortar and all samples were kept at about 55° C prior to weighing the aliquots for analysis.

Figure 3 is given in order to note whether changes occur in the chemical composition of the navel orange fruit that may be attributed to the time of blossom opening. The percentage of calcium in the dry matter of the peel and pulp tend to rise as the blossoming season progresses. The graph for the percentage of magnesium in the dry matter of the peel is similar to that for magnesium in the pulp. The percentages of potassium in the dry matter of the peel generally decrease as a result of later blossoming. In the pulp the percentage of potassium increases and then decreases as the blossoming season progresses. It is reasonable to assume because of the large content of potassium in the pulp, that toward the close of the blossoming season potassium supplies may reach a low level.

The fresh weight of the peel of the (200 size) tagged navel orange fruit as a per cent. of the fresh weight of the whole fruit was determined on all except those fruit that resulted from the blossoms of April 22, 1947. As the blossoming season progressed these values were: 27.25, 28.95, 29.74, and 29.14 per cent. respectively. As shown in figure 3, calcium in the fruit was also shown to increase as the date of blossom opening advanced.

The percentages of the 104 blossoms tagged at each successive date in navel orange trees and that resulted in fruit at harvest time were: 0, 6.7, 8.6, 9.6, 16.3, and 28.8 per cent. respectively. These are relatively low values when compared with the results obtained for Valencia orange blossoms that will be discussed next.

Valencia orange fruit from tagged blossoms

The Valencia orange crop was picked on June 17, 1948, more than one year after the blossoms were tagged. For a time at least, the one crop of fruit is in competition with the succeeding or the previous crop. Only the crop from the blossoms produced in 1947 will be considered here and it will be divided into two parts; namely, that which was sprayed with 8 p.p.m. of 2,4-D on September 4, 1947, and that which was not sprayed.

No 2,4-D SPRAY.—The number of Valencia orange fruits (and their sizes) produced from 40 blossoms tagged on successive dates during the blossoming season were: March 31, 0; April 8, 2 fruits (2 of size 200); April 15, 4 fruits (4—200's); April 22, 6 fruits (2—288's and 4—200's); April 29, 9 fruits (3—288's and 6—200's); and May 6, 20 fruits (4—288's, 15—200's, and 1—150). The fresh weights of the fruits produced from forty blossoms tagged on the various successive dates were: 0, 279, 558, 770, 1155, and 2700.7 grams respectively, whereas the percentage of blossoms that produced fruit that survived until picking time were: 0, 5, 10, 15, 22.5, and 50 per cent. respectively. More fruit therefore was produced from the tagged blossoms as the blossoming season progressed.

The blossoms of May 6, 1947, were 50 per cent. efficient in producing fruit. It is likely that the blossoms produced after May 6, 1947, were equally if not more efficient. This is a very high productivity for a certain relatively narrow or limited phase of the blossoming period, which in its overall picture often appears extremely wasteful and inefficient. Attention has been called to the possible beneficial effect of higher temperatures toward the latter part of the blossoming period.

WITH 2,4-D SPRAY.—Sixty-four blossoms were tagged on each sampling date beginning with that of March 31, 1947. On September 4, 1947, the fruits were sprayed with 8 p.p.m. 2,4-D and on June 17, 1948, the fruits were picked.

The number of Valencia orange fruits (and their sizes) produced from 64 blossoms tagged on successive dates during the blossoming season were: March 31, 0; April 8, 4 fruits (1 of size 288 and 3 of size 200); April 15, 8 fruits (3—288's and 5—200's); April 22, 15 fruits (1—288, 11—200's, and 3—150's); April 29, 17 fruits (7—288's, 9—200's, and 1—150); and May 6, 34 fruits (1—344, 7—288's, and 26—200's). The fresh weights of the fruits produced from blossoms open at successive dates were: 0, 524.5, 1015.5, 2193.1, 2181.7, and 4444.7 grams respectively. The number of blossoms that produced fruit at picking time when calculated as a per cent. of the number of blossoms tagged on the successive dates were: 0, 6.3, 12.5, 23.4, 26.6, and 53.1 per cent. respectively. These percentages are only slightly higher than when no 2,4-D spray was applied. The above results again show the increased fruit production from blossoms that open late in the blossoming season.

The prevailing fruit size was 200's regardless of the 2,4-D spray and fruit of this size were used for analysis. The fresh weights of the peel of the 200 size fruit as a per cent. of the fresh weights of the whole fruit were: for non-sprayed fruit, 25.79, 27.40, 28.74, 29.67, and 29.21 per cent. and for 2,4-D-sprayed fruit, 27.74, 29.97, 28.07, 28.07, and 28.40 per cent. respectively for successive tagging dates that were productive of fruit.

The fruit produced from the blossoms of April 8, 1947 (the first tagged blossoms of Valencia orange to yield fruit at picking time) had the lowest percentage of peel. This is in agreement with the results obtained with Washington navel orange fruits obtained from the earliest fruit-producing tagged blossoms.

Composition of Valencia oranges

Figure 4 shows the changes in the peel and pulp of Valencia orange fruits (sprayed or non-sprayed with 2,4-D) that were produced from blossoms that opened on various successive dates. All fruit used were of the 200 size in order to deal primarily with the age of the fruit and the 2,4-D spray. The percentages of calcium, magnesium, and potassium in the dry matter of the pulp tended to increase, the later the blossom-opening occurred in the blossoming season. The graphs for the 2,4-D-sprayed fruit

in most cases were smoother than those of the non-sprayed fruit. The percentages of potassium in the dry matter of the pulp of 2,4-D-sprayed fruit in most cases were higher than in that of non-sprayed fruit. The

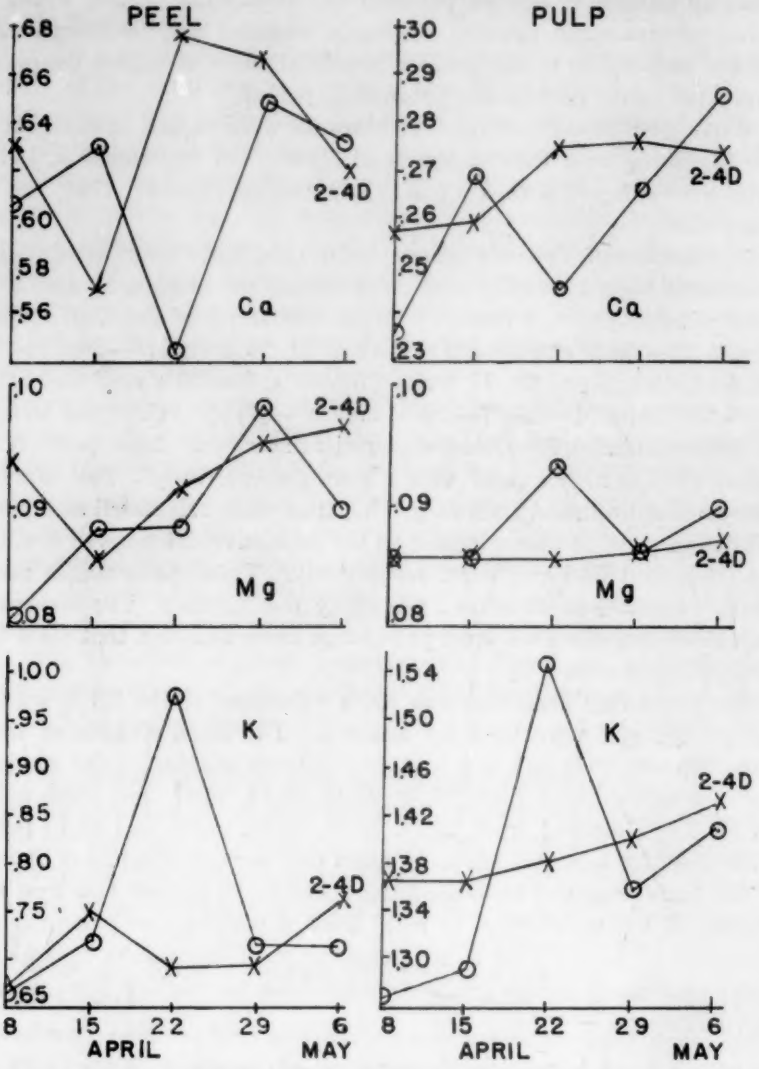

FIG. 4. Percentages of calcium, magnesium, and potassium in the dry matter of the peel and pulp of Valencia oranges (size 200) produced from blossoms that opened on successive dates in 1947. The mature crop was picked on June 17, 1948. x, trees sprayed September 4, 1947 with 8 p.p.m. 2,4-D; 0, trees not sprayed.

percentages of calcium and potassium in the dry matter of the peel and pulp of non-sprayed fruit produced by the blossoms of April 22, 1947, show the most marked departure from the values that might be expected.

Factors in fruit production

The factors operative in fruit production are many and interrelated. Here consideration is primarily directed to the effect of the date of blossom-opening during the blossoming season on the production of fruit at picking time. The first blossoms that open were assumed to have the best chance of producing a mature fruit as a result of the large content of nitrogen, phosphorus, etc., available. Under the climatic conditions and nutrition levels prevailing in 1947 and 1948 the first open blossoms had the poorest and the last open blossoms the best chance of developing into fruits that survive until picking time.

In studies of fruit production, in addition to the factor of temperature in the setting of fruit it appears desirable to include a consideration of the leaves.

FIG. 5. The spring (first) cycle of growth of an orange tree. The blossoms are borne on pedicels in the axils of the leaves. Leaves may be rudimentary or absent as in the lower two blossoms in the second shoot from the left. The leaves are in the actively growing stage as the blossoms begin to open. Most frequently the terminal blossom is the first to open. The spring cycle has both phases of growth present: vegetative (leaves and shoot) and reproductive (blossoms).

An orange blossom is ordinarily borne on a pedicel in the axil of a leaf in the spring (first) cycle of growth. Figure 5 illustrates the nature of the blossoming in orange trees. Considerable variation may occur as to the number of leaves and blossoms and the length of the pedicels.

When the first blossoms open, the leaves are very young and conceivably compete with the blossoms in their nutrition to the extent that few, if any, of the first blossoms that open ever set and produce mature fruit. Young leaves on any cycle of growth are dependent during much of their early stages of growth on reserves especially of the immediately preceding mature growth cycle.

When a citrus culture medium is deficient in certain nutrient elements

the young leaves are able to survive for varying periods at the expense of the older leaves. The leaves also can satisfy their moisture and certain nutrient requirements by withdrawals from the fruits. Citrus cuttings with attached fruits can be rooted and grown vegetatively while the reproductive phase is marked by atrophy.

Young leaves have a high priority on the mobile food supplies within the tree and the nutritional requirements of young leaves vary throughout the period of their maturation (**4**). As the leaves approach full size, though thin and immature, they become less parasitic and increasingly contribute to the development and setting of the blossoms, until as high as 50 per cent. or more of the blossoms that open are productive of fruit at picking time.

Results

The experimental results obtained in 1947 and 1948 present fairly conclusive evidence that the late-opening Washington navel or Valencia orange blossoms set fruit (which at maturity were of commercial grade) to a much greater extent than early-opening blossoms. As the blossoming season advances, the blossoms that open successively set more fruit that remain attached until fully mature. As a consequence, the various oranges on each tree may differ a month or much more in their ages according to the relative time of their blossom opening. The various ages of individual fruits in each tree may in part account for or contribute to some of the factors involved in fruit quality such as granulation, vitality, etc. Heretofore orange fruits have been assumed to be of approximately the same age. In the pollination of some varieties of oranges for breeding purposes, the above information may also be of value in obtaining fruit.

In order to know in the present investigation whether the failure of the first open orange blossoms to produce mature fruit was due to the loss of the blossom or of their fruit when nearly mature, frequent observations were made. Then careful estimates were made on June 6 and again on August 29, 1947, of the fruit produced from blossoms that opened on various dates in 1947. These estimates indicated distinctly that if fruit had been set, they had abscissed before June 6, for the results obtained at that time were in full agreement with those obtained at the time of harvest. Even prior, therefore, to June 6, 1947, it was observed that abscission of many of the first blossoms had occurred and that late opening orange blossoms set fruit to a much greater extent than early opening blossoms.

These results with orange blossoms are in agreement with the data obtained by LAMMERTS (**5**) for the setting and maturation of avocado fruits. In these pollination studies for the spring season of 1942 it was concluded that avocado fruit setting increases toward the end of the blooming period. In the case of citrus, pollination is not essential for Washington navel orange production whereas it is sometimes considered advisable for Valencia orange production.

It was further noted by Lammerts that the entire blossoming period occurred during very adverse temperature conditions. Despite this, the best chance of securing adequate progenies was to be had toward the end of the blossoming period.

Another observation that was emphasized by Lammerts was that avocado blossoms did not begin to set fruit on any of the used varieties until the new leaves were well developed. Temperature and nutritional factors were considered as being very important in these avocado studies, and for the relative importance of each, further study is required.

One possible explanation (as previously stated) for the better setting of late opening orange blossoms is the decreasing competition of maturing

TABLE II

AVERAGE MAXIMUM AND MINIMUM TEMPERATURES (FOR THE PERIODS BETWEEN AND INCLUSIVE OF THE DATES AT WHICH BLOSSOM-TAGGING OCCURRED) IN RELATION TO THE PERCENTAGES OF BLOSSOMS THAT RESULTED IN MATURE FRUIT

Period 1947	Washington navel orange			Valencia orange		
	Aver. max. temp. (°F)	Aver. min. temp. (°F)	Fruit as per cent. of tagged blossoms	Aver. max. temp. (°F)	Aver. min. temp. (°F)	Fruit as per cent. of tagged blossoms†
March 13 to 19*	78	44	0	.....	.....	.....
March 19 to 25	69	47	6.7	.....	.....	.....
March 25 to 31	75	47	8.6	75	47	0
March 31 to April 8	72	42	9.6	72	42	5.7
April 8 to 15	87	46	16.3	87	46	11.3
April 15 to 22	81	50	28.8	81	50	19.2
April 22 to 29	.....	.....	.....	71	49	24.6
April 29 to May 6	.....	.....	.....	91	52	51.6

* March 19 was the first blossom-tagging date.
† Averages of percentages for sprayed and unsprayed trees.

young leaves (on the same cycle of growth) with the blossom development. A second and perhaps equally if not more important hypothesis is that of favorable temperatures at the time of blossom-opening.

The Valencia orange blossoming period occurred about two weeks later than that of the Washington navel orange and therefore had the benefit of somewhat higher temperatures. This may in part be responsible for the greater set of fruit in the case of Valencia orange. In a large number of comparable orchards of the two varieties grown at this Station at Riverside, California, the yields of the Valencia orange trees exceed those of the Washington navel orange. Temperature is known to be very important in the earliness of bloom and very likely is also an important factor in the setting and holding of citrus fruit, although at present its analysis is difficult.

When the data are considered in relation to the temperatures (table I) and are arranged according to the average maximum and minimum temperatures for short periods just prior to and including the dates of blos-

som tagging (table II) the effectiveness of temperature becomes more apparent. It therefore appears that the average maximum temperature is very important for an increase in set when the average minimum temperature change is small and that at the average minimum temperatures of 49° F or above, the set is greatly increased. As in the production of vegetative and reproductive phases in the same growth cycle in orange trees, an interrelation may exist between blossom set and fruit production on the one hand and temperature and nutrition on the other.

Summary

Orange blossoms in the spring of 1947 were opening in the orchards of southern California during a season of about two months. The assumption was made that since the first blossoms to open contain a high nitrogen and phosphorus content and would be the first to draw on the reserve food supplies, that they would have the best chance of developing into mature fruit.

A study of how orange blossoms are produced was an aid in understanding the fruit production in orange trees. Orange blossoms are not a separate growth arising from mature tissues. They are but a part of the leafy young shoot or spring cycle of growth. The blossom usually occurs on a pedicel in the axil of a very young and developing leaf and the first blossoms to open have the greatest competition in their nutritional processes because of these young leaves.

Until such leaves lose much of their dependence upon the parent or previous growth cycle and in turn make increasing contributions to their own and possibly somewhat to the nutrition of the open blossom, the early-open blossom has little or no chance of developing into a mature fruit. In addition the early-open blossoms frequently appear before the danger of low minimum temperatures has passed and at present the evaluation of the factors of temperature and nutrition is difficult.

Freshly open blossoms on Washington navel and on Valencia orange trees were tagged on various dates during the blossoming season. In this way it was possible to know at picking time which blossoms contributed most to the production of fruit.

Instead of the first open-blossoms in 1947 having the best chance of survival and producing fruit in 1948, they were found to have the poorest or no chance at all.

None of 104 (open) navel orange blossoms tagged on March 19, 1947, contributed any fruit whatsoever to the mature crop. Open blossoms of navel orange tagged on successive 1947 dates (104 blossoms on each date) produced the following mature fruit at picking time on January 6, 1948: March 25, 7 fruit; March 31, 9 fruit; April 8, 10 fruit; April 15, 17 fruit; and April 22, 30 fruit respectively.

Oranges of equal size from early-opening blossoms had somewhat less peel than those from late-opening blossoms.

The percentages of open navel orange blossoms (tagged at various successive dates) that resulted in mature fruits were: 0, 6.7, 8.6, 9.6, 16.3, and 28.8 per cent. respectively.

Oranges of the same size but of different degrees of maturity were analyzed to note the changes that take place with increasing age. The oranges of a tree vary considerably in age, and therefore gradations of granulation, vitality, etc., are to be expected. Plant breeding tests on orange blossoms may be accompanied by different degrees of success in some varieties, dependent on the date of blossom opening.

When Valencia oranges produced from tagged blossoms that opened at various dates were sprayed with 2,4-D in September, the effect on the crop was slight. The percentages of open Valencia orange blossoms (tagged at various successive dates) that resulted in mature fruits were: for non-sprayed fruits, 0, 5, 10, 15, 22.5, and 50 per cent. and for 2,4-D-sprayed fruits, 0, 6.3, 12.5, 23.4, 26.6, and 53.1 per cent. respectively.

The production of oranges at the Riverside location, as seen from the results of open blossoms tagged on various successive dates, increased as the blossoming season progressed.

Blossom-thinning, to improve fruit quality at the expense of fruit quantity or to regulate the bearing habit of orange trees, requires a knowledge of the relation of the various dates of blossom opening to the production of the crop. This is especially true when the blossom-bearing spring cycle of growth is very short and it is difficult to distinguish the relative order of blossom opening along the leafy twig.

Two conditions, that of reduced competition in nutrition and of increased effectiveness of favorable temperatures, are considered as possible causes for the greater production efficiency of late-opening blossoms.

CITRUS EXPERIMENT STATION
RIVERSIDE, CALIFORNIA

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A STUDY OF CHEMOTROPISM OF POLLEN TUBES *IN VITRO*

TSUNG-HSUN TSAO[1]

(WITH THREE FIGURES)

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Introduction

Although much has been added to our knowledge concerning the requirements for pollen germination and pollen tube growth in recent years (**1, 2, 4, 10, 17, 18**), very little progress has been made in studying chemotropism of pollen tubes. Literature dealing with this subject prior to 1924 has been reviewed by BRINK (**3**). There is an obvious lack of agreement among the different workers as to: (a) whether chemotropism of pollen tubes to pistil parts exists at all; and (b) if so, the role that chemotropism plays in directing the pollen tubes toward the egg. The present investigation is an attempt to further our knowledge of this process.

Methods

The plant materials used during the course of this investigation came chiefly from the greenhouses of the Botany Department and the garden of the Horticulture Department of the University of Wisconsin. An attempt was made to use as many plant species as were available and had been reported in the literature to show positive pollen tube chemotropism, with a view to confirming and testing the earlier results. Of these plants, *Hippeastrum Johnsoni*, a hybrid, was found to be the most favorable material for the study of chemotropic response because of its large stigma and pollen grains. Its use, however, was limited by its restricted flowering season.

The medium found most suitable for culturing pollen tubes of most plants contained 1% agar, 10% sucrose and yeast extract at a concentration of 100 p.p.m. Yeast extract was used since, according to BRINK (**3**), it decidedly accelerated pollen germination and promoted pollen tube growth.

While still hot, the agar medium was poured into a Petri dish to form a layer about 4 mm. thick. Pistil parts including whole ovules and sliced stigma, style, placenta, and ovary wall were imbedded near the surface when the agar started to gel. Pollen grains were spread with a camel's hair brush around the pistil slices after the medium had hardened. For low power examination the Petri dishes were inverted; cover slips were placed directly on the agar plate for high power examination.

Pollen grains of most species germinate within one to two hours, and since observations were usually completed within two hours, no special

[1] This work was done at the University of Wisconsin, where the author was a University Fellow in the Department of Botany.

sterilization procedures were employed. All experiments were carried out at room temperature which did not vary more than two degrees from 25° C.

The preliminary investigation consisted of an attempt to determine whether chemotropism could be demonstrated in vitro. After this phenomenon was demonstrated, two methods of experimentation were employed. One involved determining the nature of the active factor which is present in the pistil parts; the other was an attempt to duplicate the effect of the natural tissue with known substances.

The technique found most suitable for the application of known substances involved preparation of agar containing known concentrations of the substance used. Two-millimeter cubes were cut from this solidified agar. These cubes were placed on the surface of the culture plates in the same fashion as the pistil parts. By this method, a number of substances in varying concentrations were tried. These included sucrose, White's medium containing inorganic salts (**21**), indole-3-acetic acid, organic acids, amino acids, proteins, and various other nitrogen-containing substances. Some of these substances had been reported to have pronounced effect on the stimulation of pollen tube growth. Some are believed to be effective in inducing chemotropism of pollen tubes, while others are essential in plant metabolism.

Various attempts were made to isolate the active substance or substances from the pistil parts. These included extraction with water, ether, and 95% alcohol. The tissues were also placed on agar blocks at different pH levels, with the idea that the active substance might diffuse out into the agar. The agar blocks were then tested for activity.

In order to determine some of the properties of the active factor, the active tissues were subjected to various treatments before being used. To study the thermo-stability of the active factor, the tissues were heated at 100° C. To get some idea of the molecular size of the substance, pieces of the tissues were wrapped in commercial cellophane membranes, and then imbedded in the agar of the test plates. Activity was also determined after other pieces of the tissues had been hydrolyzed with 5% HCl and then neutralized with NaOH.

Results

The occurrence of chemotropism

Among the 36 species of plants tested (see table I and text below), only a small number showed pollen tube chemotropism under the specified experimental conditions. Those which showed positive results are listed in table I. In no case was chemotropism shown to pistil parts of flowers of other species. For example, pollen tubes of Hippeastrum were indifferent to the stigma of *Vinca major*, and pollen tubes of *Vinca major* were indifferent to the stigma of Hippeastrum. Pollen tubes of *Primula obconica* showed no re-

sponse to the stigma of Antirrhinum, nor did the tubes of Antirrhinum respond to the stigma of Primula. Pieces of young embryo, seed coat, sepal, petal and leaf of *Antirrhinum majus* (2N) did not attract pollen tubes of that species.

There is a maximum effective distance beyond which the influence of the active tissue does not extend. Beyond this distance, the pollen tubes grow at random. This is shown in figures 1 and 2. The effective distance in *Antirrhinum majus* and Hippeastrum was measured and was found to be 1 – 1.5 mm. This is in agreement with observations by MOLISCH (13) in *Narcissus Tazetta*. The agreement of the measurements of the effective distances presumably indicates similar diffusion patterns and therefore may mean the effective substances in the two cases have approximately the same molecular weight.

If, while still growing, the tubes were turned gently with a camel's

TABLE I

PLANTS IN WHICH POLLEN TUBE CHEMOTROPISM OCCURRED

PLANTS	TISSUES THAT ATTRACTED POLLEN TUBES
Hippeastrum Johnsoni	Stigma only
Hippeastrum puniceum	Stigma only
Narcissus Tazetta	Stigma, ovule and placenta
Narcissus poeticus	Stigma, ovule and placenta
Antirrhinum majus (2N)	Ovule and placenta always; stigma and cut end of style under certain conditions
Antirrhinum majus (4N)	Ovule and placenta, other parts uncertain
Lilium superbum	Stigma, cut end of style, placenta and ovule
Paeonia sp.	Ovule and stigma
Hemerocallis fulva	Stigma, placenta and ovule; not shown late in the season
Hemerocallis sp.	Stigma, placenta and ovule; not shown late in the season

hair brush, they would again grow toward the source of the chemotropic stimulus.

Early in the investigation, it was found that the pollen tubes of *Antirrhinum majus* showed chemotropic response only to ovules and placentae. However, later in the season chemotropic response both to the stigma and to the cut end of the style was occasionally observed. Since the medium used was the same and the laboratory conditions did not change appreciably, some change in the plant itself is suggested. Since these plants were growing out of doors, possible causal factors are the age of the plants, day length, and temperature, singly or in combination.

A study of the effect of age of Antirrhinum flowers was made. Pollen grains obtained in July from freshly dehisced anthers were sown around the imbedded pistil parts of flowers and flower buds of different ages. The presence or absence of attraction is shown in table II. The placenta and the ovule attracted the pollen tubes at all stages of their development, while the stigma and style affected the pollen tubes only if they came from very young buds or from mature flowers after anthesis. The age of the

flower, therefore, seems to influence the induction of chemotropic response in Antirrhinum, but it does not seem to be the only factor involved. That other factors are also involved is indicated in Hemerocallis (table I) where the stigma, placenta and ovule all strongly attracted the pollen tubes early

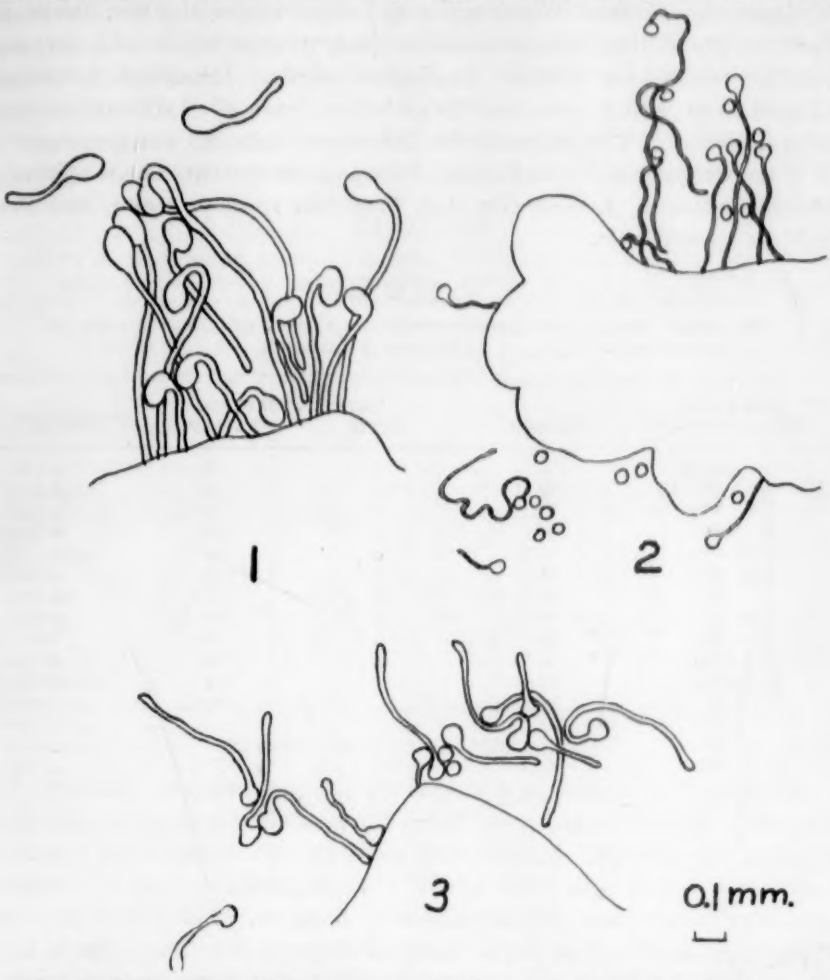

FIG. 1. Pollen tubes of Hippeastrum which have been attracted by a stigma piece of the same species. The maximum effective distance in this case is approximately 1.3 mm.

FIG. 2. Pollen tubes of *Antirrhinum majus* showing chemotropic response to a piece of placental tissue of the same species. The maximum distance is at least 0.7 mm.

FIG. 3. Pollen tubes of Petunia hybrida growing in all directions regardless of the imbedded stigmatic tissue.

All drawn with camera lucida to the same scale.

in the season, but not after the beginning of July. The season for this seasonal change remains to be discovered.

In most of the plants investigated, pollen grains grown on the artificial

medium in the vicinity of imbedded pistil parts sent out their tubes in all directions quite regardless of the presence of the imbedded tissue (figure 3). This was true with the following species: *Primula malacoides, Primula kewensis, Petunia hybrida, Vinca major, Muscari botryoides, Scilla sp., Tradescantia paludosa, Pisum sativum, Tulipa Gesneriana var. Darwinia, Eucharis grandiflora, Lilium eximium* (only stigma was tried), *Papaver orientale, Oenothera biennis, Rudbeckia tricolor, Impatiens balsamina, Allamanda sp.*, and *Lupinus sp.* In all but one case, all of the various pistil parts were used. The pollen of the following plants did not germinate at all in the medium used: *Fuchsia sp., Pelargonium sp., Taraxacum officinale, Abutilon hybiscus, Senecio cruentus, Browallia speciosa major*, and *Dendrobium thyrsiflorum.*

TABLE II

THE EFFECT OF AGE OF FLOWERS ON THE INDUCTION OF CHEMOTROPISM IN *Antirrhinum majus*. + = PRESENCE 0 = ABSENCE + ? = DOUBTFUL

AGE (DAYS FROM ANTHESIS)	CHEMOTROPISM TO: STIGMA	STYLE	PLACENTA	OVULE
8 before	+	+	+	+
5 "	+	+	+	+
4.5 "	0	0	+	+
4 "	0	0	+	+
3 "	+ ?	0	+	+
2.5 "	0	+ ?	+	+
2 "	0	0	+	+
1.5 "	0	+	+	+
1 "	0	+ ?	+	+
1 after	+	+	+	+
2 after	+	+	+	+

TRIALS WITH KNOWN SUBSTANCES

A series of substances in a wide range of concentrations (0.01, 0.1, 1.0, 10.0, 100.0, 1000.0 p.p.m.) were tested for possible effects on the direction of pollen tube growth. Studies were made on *Lilium superbum* and *Hippeastrum Johnsoni* with water soluble vitamins and a number of nitrogen-containing substances. The results were negative. The experiment was repeated on *Antirrhinum majus* using 17 different substances (table III). The number of pollen tubes growing in various directions was counted and the results analyzed statistically.

In table III a plus sign indicates growth towards the substance, a minus sign growth away from it. Growth was called positive if the pollen tube pathway between pollen grain and test block was within the quadrant subtending the grain, negative if within the opposite quadrant, and neutral if within the lateral quadrants. Neutral figures are not presented in the table. All values are derived from five replicated samplings for each dilution of each substance.

It appears that in every case the numbers of pollen tubes growing in

the positive and negative directions are about the same. Application of the chi-square test showed that even in the extreme case (thiamin at 0.1 p.p.m.), the deviation from random is hardly significant.

It can be seen readily in figures 1 and 2 that, in every case of positive chemotropism induced by tissues, all the pollen tubes within the maximum effective distance grew in the positive direction. This further supports the statement that none of the substances, at any concentration tried, had a comparable effect.

To determine whether sugar might be involved in the chemotropism, pieces of Hippeastrum stigma were imbedded in plain agar, and adjacent

TABLE III

EFFECT OF SOME WATER SOLUBLE VITAMINS AND SOME PHYSIOLOGICALLY IMPORTANT NITROGENOUS SUBSTANCES ON THE DIRECTION OF POLLEN TUBE GROWTH OF *Antirrhinum majus*. FIGURES REPRESENT THE NUMBER OF TUBES GROWING IN EACH DIRECTION (SEE TEXT).

SUBSTANCES	CONCENTRATION (P.P.M.)											
	.01		.1		1		10		100		1000	
	+	−	+	−	+	−	+	−	+	−	+	−
Asparagine	19	18	21	26	22	16	18	14	18	22	15	12
Urea nitrate	21	19	15	19	18	14	15	13	16	19	20	20
Urea	19	22	20	17	21	16	14	18	17	19	19	15
Na ureate	18	16	17	18	17	12	20	15	15	17	18	23
Choline	14	17	19	15	12	18	15	15	25	20	22	18
Adenine	15	14	19	14	13	12	17	17	29	22	14	15
Guanine	14	17	18	16	16	15	19	15	17	13	18	11
Uracil	20	24	16	20	14	17	19	13	20	18	26	23
Barbituric acid	26	17	16	14	17	14	14	18	18	15	21	23
Na nucleate	14	15	25	20	15	8	18	13	21	14	23	12
Thiamin	21	28	24	12	17	17	16	11	13	18	.....	.....
Riboflavin	24	15	22	13	25	24	16	17	18	11	.....	.....
Pyridoxine	26	18	17	12	23	21	15	17	26	19	.....	.....
Niacin	17	18	27	21	30	24	23	11	12	13	.....	.....
Nicotinamide	16	20	14	18	22	12	14	19	16	11	.....	.....
Inositol	14	20	18	20	20	16	17	16	20	13	.....	.....
P-Aminobenzoic acid	22	13	23	19	18	18	17	16	13	21	.....	.....

to these pieces, blocks of agar were removed and replaced with equal size blocks containing 10% sucrose without stigma pieces. Hippeastrum pollen grains were sown along the line separating the sucrose-containing block from the agar containing the stigma. All the tubes grew to the stigma side, none to the sugar side. Of course this does not exclude the possibility that the stigma contains a higher concentration of sugar than 10%, but this is very unlikely. In the same way, the major and minor mineral elements for plant growth were found to be ineffective in inducing chemotropism, as were also yeast extract and casein. (The casein, which is only sparingly soluble in water, was tested by imbedding crystals directly in the agar.)

Organic acids and some of their salts were tried on pollen of Hippeas-

trum by PFEFFER's (15) capillary method. Concentrations of 100 p.p.m. of succinic acid and its sodium salt, dl- and l-malic acids, fumaric, citric, lactic, pyruvic, glutaric, and glutamic acids were used. None of these showed any effect. Similar results were also obtained with the same plant material using an adaptation of the penicillin assaying cylinder method (5) and acids at a concentration of 200 p.p.m. Peptone, over a concentration range from 2% to 0.002% and indole-3-acetic acid at 10 p.p.m. did not have any effect on the direction of pollen tube growth of *Hippeastrum*.

ATTEMPTS TO ISOLATE THE ACTIVE FACTOR

Various methods were used in an attempt to isolate the active factor from the pistil tissue. Agar was made up at different acidities (pH 4.0, 6.9, 9.1) and allowed to solidify. Lobes of fresh stigma tissue of *Hippeastrum Johnsoni* (the stigma is tri-lobed) and masses of ovules of Antirrhinum were placed directly on the agar surface. Cubes of agar just large enough to hold the tissue were then cut out. These small cubes were put on a pad of moistened filter paper in a covered Petri dish. Diffusion into the agar was allowed to take place at room temperature for three and one half hours. The tissues were then removed and the agar blocks inverted on another culture plate containing solidified agar medium, so that the surface with which the tissues had been in direct contact touched the surface of the culture medium. Pollen grains were spread around the cubes in the usual manner. The results indicated no transfer of an active factor to the agar.

Positive results were finally obtained with *Lilium superbum* using 3% agar at pH 6.9 instead of the 2% as above and allowing diffusion to continue for five hours. The pollen tubes were markedly attracted by these cubes. Apparently part of an active factor existing in the tissue had diffused out into the agar blocks.

Water extractions were prepared in the following way. Fresh stigma tissue from five flowers of Hippeastrum was ground in a mortar with 2 ml. of distilled water and then filtered through filter paper. The filtrate was subjected to activity tests by several methods. In each test the filtrate failed to induce positive chemotropism. Similarly, the filtrate from ground ovules of eight flowers of Antirrhinum did not show any activity. The extraction was repeated by grinding the tissue with a little $CaCO_3$ to neutralize any acid which might have been produced from the tissue breakdown. The filtrate was still not active.

The active tissue was also ground with ethyl ether. Filter paper strips were soaked in the filtrate, the ether was allowed to evaporate completely, and the filter paper strips were used for the activity test. The pollen tubes were found to be indifferent to them. A trial with 95% ethyl alcohol extracts gave the same result. Although the filtrates were not active in any of the above cases, the mashed residues of the parts used remained active.

Stigmas of Hippeastrum still retained their activity in attracting the pollen tubes after being boiled in 50 ml. of distilled water for 10 minutes. Hydrolysis with 5% HCl for 10 minutes followed by neutralization with NaOH also failed to destroy their effectiveness. However, stigmas wrapped in a commercial cellophane membrane and imbedded in agar, completely failed to attract the tubes growing around them. This indicates that the active substance could not pass through the pores of the membranes. The exact porosity of the membrane was not determined, but these facts suggest that the molecular weight of the substances must be relatively high.

Discussion of results

The above observations substantiate earlier reports on chemotropism of pollen tubes. The failure to demonstrate this phenomenon under certain experimental conditions does not necessarily mean that chemotropism does not exist in nature, nor that it is without significance in directing the growth of pollen tubes to the embryo sac.

It is true that this phenomenon can be more consistently demonstrated in some species than in others. Pollen tube chemotropism of *Narcissus Tazetta*, previously reported by Molisch (**13**), Lidforss (**7**), and Tokugawa (**20**), was verified in the present investigation. Similarly, the present findings agree with those of previous authors in the case of Antirrhinum. On the other hand, the present work did not confirm Miyoshi's results on Scilla and Primula (**12**), nor Brink's work on Hippeastrum (**3**). These discrepancies are understandable when one considers, first, that the situation varies even within a species handled under identical laboratory conditions and with the same kind of medium as was demonstrated in this study; and second, that the occurrence of pollen tube chemotropism is affected by the concentration of the culture medium, as has been shown by Lidforss (**8**). It seems likely that there are some undetermined internal and external factors which regulate production of some unidentified substance which is active in the induction of chemotropism.

Despite the failure to identify the active factor, the above studies give some idea of its properties. It must be a slowly diffusing, heat stable, water soluble substance of considerable molecular size. It is rather resistant to acid hydrolysis. Since the stigmas of Hippeastrum still retained activity after being boiled 10 minutes in 50 ml. of water, the active substance must be effective in very small concentrations.

The comparable effect of sodium malate both on fern spermatozoids (**13**) and on the pollen tubes of Antirrhinum (**3**) suggests that the active substance may not be specific. This suggestion is supported by the observation of some investigators that pollen tubes show chemotropism to the stigmas and ovules of quite distantly related species and is further strengthened by the fact that the maximum distance observed by Molisch in *Narcissus Tazetta* (**13**) and by the present author in Hippeastrum and in Antirrhinum was approximately the same.

The mechanism of pollen tube chemotropism does not seem comparable to the chemotactic movement of lower organisms, such as bacteria and some unicellular algae, and of the spermatozoids of fern. In those cases, the whole cell moves, usually by means of cilia or flagella, toward the chemical stimulus. It is, however, more or less like the chemotropism of hyphae from germinating fungus spores (**11**). Knowledge of this phenomenon is also scant. Positive chemotropism of seedling stems and roots of higher plants has been reported (**6, 9, 14, 16**), but the mechanism involved may be even more complicated. Since, in the present case, we are dealing with a single cell rather than a multicellular organ, like stem and root, it would appear that the bending response of the pollen tube might occur as a result of unequal growth of the wall on two sides of the tube. In pollen tubes the wall is composed mainly of pectic substances. If the growth of this wall varied inversely with the concentration of the active substance, in other words, if the substance inhibited the extensibility of the wall material, directly or indirectely, the observed response would result.

The ineffectiveness of the water extract of the tissue described above may be explained by assuming that some interfering wound substance is released. It would be difficult to explain otherwise, since the effective substance is water soluble. The explanation for the inactivity of the residue from ground tissue is possibly due to the fact that the active factor is continuously formed from the tissue, as in the case of auxin.

That the substance involved in chemotropism is probably not nutritive in nature is seen from the experimental results which show that substances known to be essential for tube growth failed to induce chemotropism. Therefore, "chemotropism" is to be distinguished from "trophotropism," which, as defined by STRASBURGER (**19**), is the movement of an organism or an organ toward a food source.

Summary

1. Of 36 species of plants studied, nine showed positive pollen tube chemotropism to their respective pistil part or parts, 20 did not, and the pollen of the other seven species did not germinate at all. The medium contained 10% sucrose, 1% agar and 100 p.p.m. of yeast extract in distilled water.

2. The chemotropism of pollen tubes was shown to be related to the developmental stages of the flower in Antirrhinum. It also seemed to vary with some undetermined internal or external factors.

3. Of all the pure substances tried, including sucrose, the major and minor essential minerals, water soluble vitamins, indole-3-acetic acid, organic acids, peptone, casein and other nitrogen-containing substances, none showed any effect in attracting or repelling the pollen tubes of any species. Compressed yeast and yeast extract also failed to show any effect. Tissues other than pistil parts, such as young embryos, seed coats, sepals, petals

and leaves of *Antirrhinum majus* did not attract pollen tubes of the same species.

4. The active factor in the stigma of *Lilium superbum* was shown to diffuse out from the tissue into an agar block, and subsequently from the block into the culture medium to exert its influence.

5. The active factor in all cases studied was shown to be a slowly diffusing, heat stable, water soluble substance of considerable molecular size. It must be effective in very low concentrations.

6. As an explanation of the mechanism of pollen tube chemotropism, it is postulated that the active substance may directly or indirectly inhibit the extensibility of the wall material of the tube, thus controlling the direction of growth.

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THE PLANT RESEARCH INSTITUTE
THE UNIVERSITY OF TEXAS
AUSTIN, TEXAS

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THE EFFECT OF TEMPERATURE UPON TRANSLOCATION OF CARBOHYDRATES IN THE TOMATO PLANT

F. W. WENT AND H. M. HULL

(WITH TWELVE FIGURES)

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One of the most controversial problems in plant physiology is that of translocation of organic materials inside the plant. This is mainly due to a lack of information. In spite of the investigations of Crafts, Curtis, Mason, Münch, Phyllis, Schumacher and many others, we need much more basic information before we will have a proper insight in this problem and before we can accept a satisfactory theory of translocation.

The recent work of WENT (**17**) had stressed the importance of the process of sugar translocation at higher temperatures, since this seemed to be the limiting process in the growth and development of tomato plants. Furthermore, it had been concluded on the basis of three different lines of experimental approach, that the Q_{10} of sugar transport was less than 1 in the tomato plant. In subsequent work more evidence was collected for the low Q_{10} of sugar translocation, not only in the tomato plant (WENT **18**, WENT and ENGELSBERG **21**, WENT and CARTER **20**), but also in the chili pepper (DORLAND and WENT **7**).

A Q_{10} smaller than 1 was contrary to the accepted view, that at low temperatures sugar translocation decreases. This view was based on the work of several investigators. CHILD and BELLAMY (**2**) had observed a greatly increased outgrowth of adventitious buds on the leaves of Bryophyllum when their petioles were chilled to 2.5 – 4 C for a few days. This seemed to indicate a greater concentration of carbohydrate in the leaves with chilled petioles, but no direct sugar determinations were carried out to support this interpretation. Therefore the results might as well be interpreted as an increased rate of movement of other bud growth factors towards the leaves. CURTIS (**4**) and CURTIS and HERTY (**5**) studied the effect of temperature of the petiole on the loss in darkness of dry material from the primary leaves of the red kidney bean (*Phaseolus vulgaris L.*) It was found that cooling the petioles to 0.5–4.5° C greatly retarded translocation of carbohydrates from the leaf blades. However, if the treatment was continued for 17–20 hours, a significant amount of transport took place. Sugar transport was also retarded when the entire plant was kept at temperatures of 0–2° C. At intermediate temperatures of 7–11° C sugar translocation appeared to be intermediate between the values found for low temperature and values for higher temperatures of 17–24° C.

Chilling of the main stem just below the first pair of leaves for two days to a temperature of 2–5° C clearly showed that the upward movement of

nutrients had been interfered with, as well as the downward movement of sugars. Analysis of the the entire plant top proved this. Chilling of the main stem had no appreciable effect on the actual amount of water movement. Plants with chilled stems bled as freely when cut above the chilled section as did the non-chilled controls, which were cut at the same relative position. In other experiments involving a chilling of the stem, the leaves would never lose their normal turgor.

KRUSEMAN (**9**), using another species of bean (*Phaseolus multiflorus* Lmk.), came to a somewhat different conclusion. His first work with cut stems, in which the iodine test for starch was used, showed decreased translocation when the petioles were kept at a lower temperature. Since the iodine test can hardly be considered accurate for quantitative determinations, Kruseman based his later experiments on dry weight. Although he concludes that in some of his experiments chilling of the petioles retards

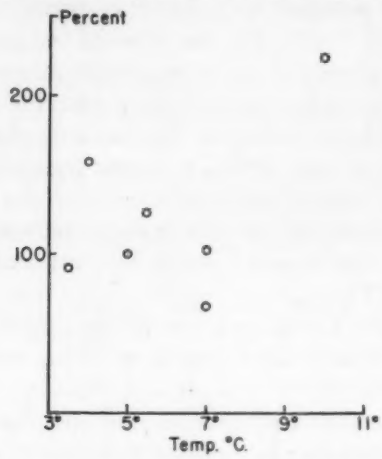


FIG. 1. Amount of translocation from bean leaves when their petioles are chilled to the temperatures shown on abscissa, when amount of translocation in the non-chilled leaves is set at 100 (data from KRUSEMAN 9).

translocation, a critical survey of his data fails to indicate a definite trend, as the correlation table in figure 1 shows, summarizing KRUSEMAN's experiments. This figure gives the dry weight of the leaves with chilled petioles as percentage of the control leaves kept at room temperature. Values based on weight changes per unit leaf area gave a similar wide scattering, with no correlation trends.

Chilling of the stem of Cucurbita to 2–4° C was found by CRAFTS (**3**) to retard exudation (considered to be phloem-exudate) from a cut stem about 50 per cent. He suggested this effect may be due to a change in the viscosity of the solution of nutrients, and that although protoplasmic streaming may be stopped at such temperatures, because of a similar increase in viscosity, it does not necessarily follow that a direct relationship exists between protoplasmic streaming and longitudinal transport of solutes.

The chemical composition of the exudate from the chilled and unchilled plants in the above experiment was not significantly different, thus showing that nothing had precipitated or crystallized out in the cooled stems.

VAN DER WEY (**15**) found that the velocity of transport of growth substance in the Avena coleoptile appears to be almost independent of temperature. At temperatures below 40–45° C however, the volume of transport varies according to temperature, much as the curve representing a life process varies. Transport at 0° C in the coleoptile is decreased to a value approaching the rate of diffusion, yet it remains polar, and on account of this, is unlike diffusion. The fact that the rate of protoplasmic streaming is greatly influenced by temperature would indicate that translocation is largely independent of this phenomenon, in the case of growth substance in Avena. Later, BOTTELIER (**1**) found that the rate of protoplasmic streaming in the Avena coleoptile is affected by temperature but little within the normal range of most experiments.

On the other hand, SCHUMACHER (**14**) by using fluorescein dye, has shown that the velocity of transport is greatly influenced by temperature, and that very little transport takes place at temperatures near 0° C. This discrepancy may be due to the fact that the channels and the mechanism for translocation are entirely different for auxin and for fluorescein. SCHUMACHER found that fluorescein is carried solely in the protoplasm, and thus its transport is probably largely through parenchyma cells.

In work on Biloxi soybean, in which the entire plant was kept at temperatures between 55° and 85° F (13–29° C), PARKER and BORTHWICK (**11**) have shown that sucrose, reducing sugars and total sugars in the leaves and stems of plants increased with a decrease in temperature during the dark period.

It was later found (PARKER, BORTHWICK and HEINZE, **13**) that Biloxi soybean plants in which the petioles were cooled during a four-day photoperiodic induction period showed an inhibition of flowering, apparently resulting from the influence of low temperature on the transport of a flower-forming stimulus from the leaves. The petioles were cooled to temperatures ranging from 3 to 25° C. In a still more recent paper (PARKER and BORTHWICK, **12**) it was demonstrated that when a leaf of Biloxi soybean is kept at various temperatures ranging between 45° and 105° F (7–41° C) during a five-day induction period, floral initiation is greatly inhibited at temperatures lower than 13° C. Such an inhibition is probably due to the temperature effect on photoperiodic reactions occurring in the leaf blade during the dark period rather than its effect on translocation of a flower-inducing stimulus from the leaf to the terminal meristems. Thus it might seem from this work that low temperature inhibits the transport of flower stimulus, but increases the rate in the case of carbohydrates. However, no conclusions should be drawn from this since only local chilling was used in two cases, and the entire plant chilled in the other case.

In the present work the rate of sugar transport is measured by record-

ing how long it takes between the moment 7 per cent. sucrose is applied to leaves and the moment that this sugar becomes effective in the root system. The activity of the root system is measured by the rate of exudation (bleeding) which requires respiratory energy (see *e.g.* VAN OVERBEEK, **16**). Since the rate of bleeding is variable even under completely controlled conditions, but varies parallel for all plants in one single experiment, the effect of applied sugar can only be measured by comparing the rate of bleeding of sugar-treated and control plants in individual experiments.

Methods

The plants used in the present experiments were all tomatoes of the San Jose Canner variety, which were grown in four-inch pots in river sand

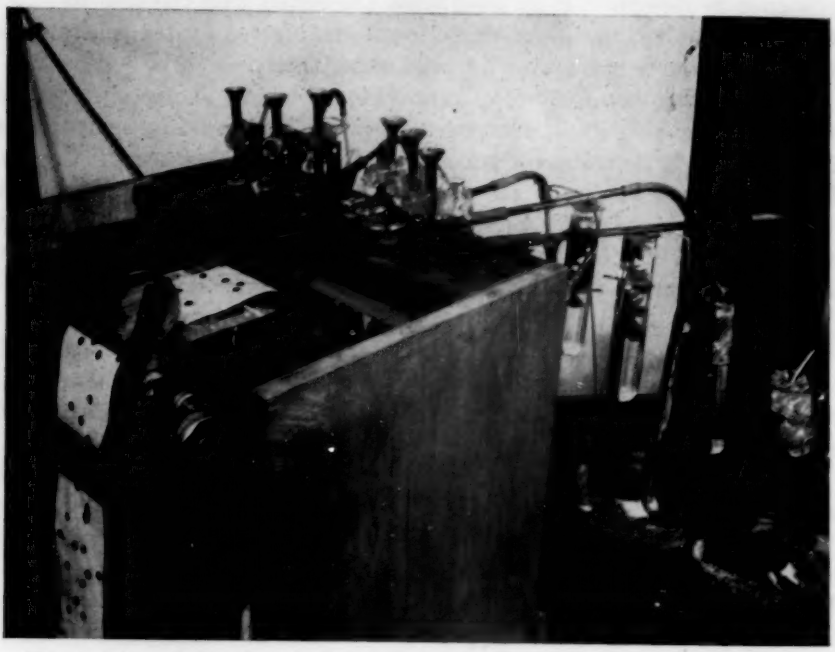


FIG. 2. One of the bleedometers used in the experiments. Six tomato plants are attached to six driptubes, whose drops fall on the strip of paper, which is pulled from right to left by the rollers at left, connected with an electric clock motor. Two leaves of each plant are immersed in test tubes; their stem is surrounded by a cooling jacket (insulating cover of jacket has been removed).

and later transplanted into seven-inch pots. They were watered three times daily with full strength Hoagland's nutrient solution, and used at an age of seven to 10 weeks, at which time they were 60–90 cm. tall.

Leaves were pinched off from the lower 22 cm. of the stem in order to allow installation of the cooling collar. This was done four days or more before the experiments in order to allow ample time for healing. It was shown (WENT and CARTER, **19**) that cutting leaves off inhibited sugar trans-

location in the stem somewhat more than when they were pinched off, and that this inhibitory effect decreased with time.

The bleedometer used was described in an earlier paper (WENT **17**). The only modification was an installation of electric resistance heaters, located so that the paper with its freshly fallen drops of dye would pass over them. This dried the drops more efficiently than the capillary tubes described in the earlier paper. Two bleedometers for six plants each were used, so that recordings on 12 plants could be made simultaneously.

Figure 2 shows one of the bleedometers. The recording paper moves between wooden cylinders, of which the lower one is rotated with a small electric clock motor, the cylinder being connected with the minute hand

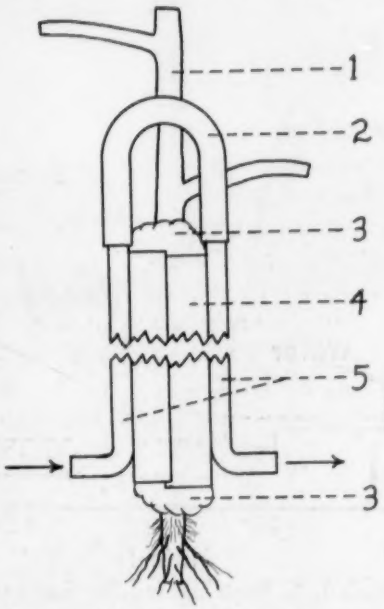


FIG. 3. Metal collar (4) as installed around tomato stem(1). Each half of the collar has a copper tube (5) soldered to it, through which the cooled water flows. The stem is held in place inside the collar by two cotton plugs (3).

shaft. Small metal points slightly protruding from the lower cylinder prevent slipping of the paper and provide time-marks. The plants are attached with rubber tubing to T tubes, one end narrowed to a drip point above the strip of paper, another, closed with a stopcock, widened to a funnel to fill the tubes. The figure shows the tomato plants in pots, with the collar attached around the stem, and two leaves immersed in large test tubes filled with sugar solution or water.

The plants were divided into three series of four plants each. The first four had a 7 per cent. sucrose solution applied to two of their leaves, and 22 cm. long collars consisting of the two halves of longitudinally split metal tubing, which were clamped around the base of their stems. Copper

tubing was soldered to both halves of these collars, through which could be circulated water at any desired temperature, thermostatically controlled to ± 0.5° C. The stems were wrapped with cotton batting at each end of the collar, before its application. The latter was then wrapped with paper toweling to provide further insulation. Figure 3 shows a sketch of one of these collars installed on the plant. Temperature drop through the series of four plants was less than 1.0° C, when working at the lowest temperatures. All temperatures indicated are the median of this 1.0° range and were taken within the collar itself. It should be stressed that the water circulated only through the copper tubing, and not between collar and stem.

The second series of four plants was treated in the same way except

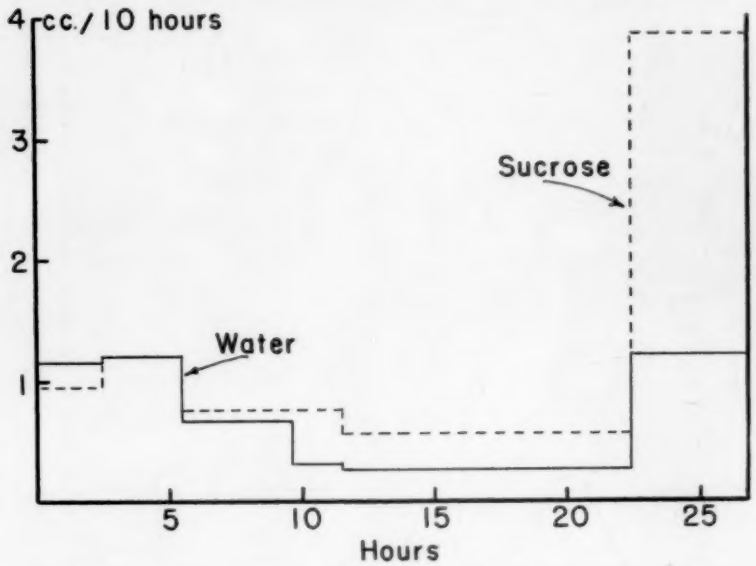


FIG. 4. Early experiment, in which the bleeding sap was collected in graduated tubes. Rate of bleeding in cc./plant/10 hours (ordinate), when two leaves were submerged in water or in 10 per cent. sucrose solution.

that no water was circulated through the collars. They therefore remained at room temperature, which was thermostatically controlled at 23.0° ± 1.0° C.

The third series acted as controls, and had two of their leaves immersed in distilled water. The stems also had collars, just as the second series.

The two leaves immediately above the collar were the ones immersed, and the plant was decapitated just above them. In this way no leaves were exposed to the air where water might be lost by transpiration. The plants were watered well at the beginning of the experiment, and the pots were constantly kept in a water bath, about 1 cm. deep, throughout the period of bleeding.

In preliminary experiments, it was found that the bleeding of sucrose-

fed plants began to increase over the controls about 11 hours after application of the sucrose, which would indicate that it takes about this length of time for transport from the leaves to the roots.

The actual rate of bleeding in drops per hour was plotted every hour for the average of each series of four or more plants. A percentage curve

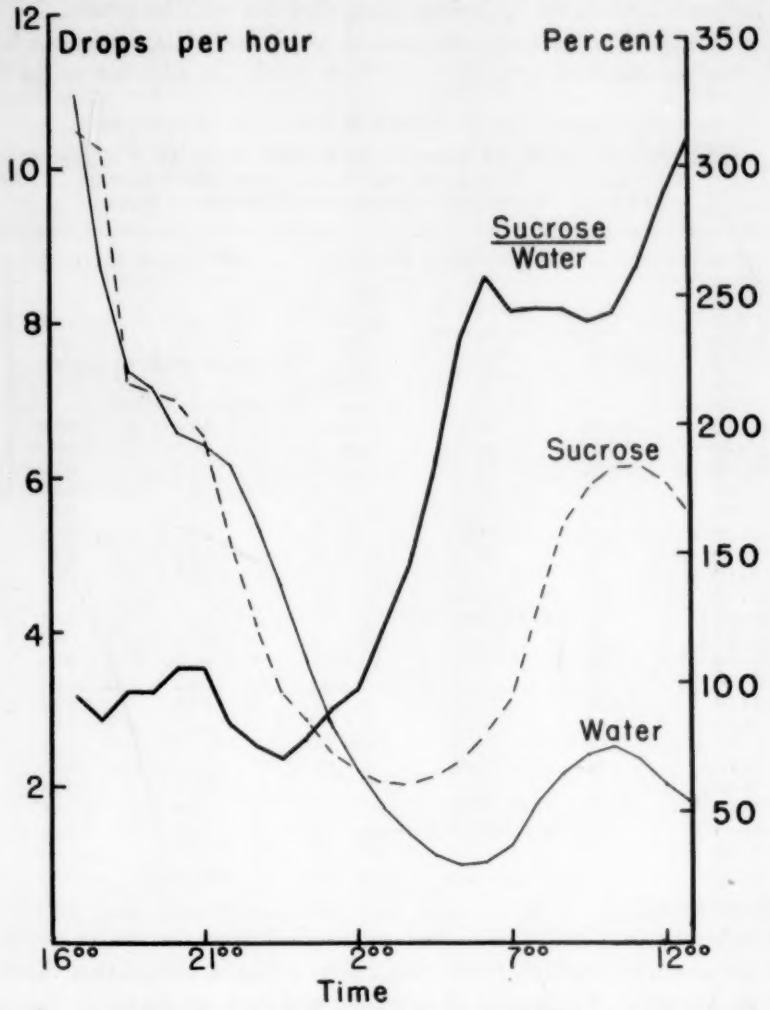


FIG. 5. Another early experiment, in which the rate of bleeding is plotted over a 21-hour period for two groups of plants, one having two leaves immersed in water, the other having two leaves in 10 per cent. sucrose.

was then made which compared the rate of bleeding of the sucrose-fed plants with the rate of bleeding of the controls.

Immediately following each experiment the roots were removed and dried for sugar analysis. Sucrose was determined on water extracts of the

material by determining the difference in reducing power of the two aliquots of each sample, one before and one after hydrolysis of the extract with invertase. Reducing power was measured by titration with ceric sulphate, according to the method of HASSID (**8**). Since reducing sugars were not evaluated, this simplification was used. The sucrose values are not influenced when the hydrolysis is carried out with invertase.

In some of the later experiments in which the collars were cooled to 5° C, four series of three plants each were used. In addition to the three

TABLE I

ACTUAL RATE OF BLEEDING OF TOMATO PLANTS WITH STEMS AT 1° C AND 24° C, WITH LEAVES IN 7 PER CENT. SUCROSE, AND THE PERCENTAGE AS COMPARED TO CONTROLS WITH LEAVES SUBMERGED IN WATER

TIME	AVERAGE RATE OF BLEEDING, DROPS PER HOUR			PERCENTAGE OF CONTROLS	
	LEAVES IN 7% SUCROSE STEMS 1° C (11 PLANTS)	LEAVES IN 7% SUCROSE STEMS 24° C (9 PLANTS)	CONTROLS. LEAVES IN WATER STEMS 24° C (7 PLANTS)	STEMS 1° C / CONTROLS	STEMS 24° C / CONTROLS
11:00	12.48	19.27	19.97	62.5	96.5
12:00	11.55	21.20	18.78	61.5	112.9
13:00	9.92	17.82	18.71	53.0	95.2
14:00	8.35	12.93	14.22	58.7	90.9
15:00	6.82	9.47	10.65	64.0	88.9
16:00	5.35	6.31	7.07	75.7	89.3
17:00	3.92	4.19	4.89	80.2	85.7
18:00	2.52	2.70	3.54	71.2	76.3
19:00	1.82	1.96	2.59	70.3	75.7
20:00	1.53	1.41	2.07	73.9	68.1
21:00	1.36	1.12	1.69	80.5	66.3
22:00	1.22	.83	1.24	98.4	66.9
23:00	1.20	.67	1.04	115.4	64.4
24:00	1.21	.57	.84	144.0	67.9
1:00	1.25	.56	.76	164.5	73.7
2:00	1.32	.60	.81	163.0	74.1
3:00	1.35	.70	.73	184.9	95.9
4:00	1.34	.78	.73	183.6	106.8
5:00	1.24	.82	.74	167.6	110.8
6:00	1.16	.90	.77	150.6	116.9
7:00	1.09	1.00	.77	141.6	129.9
8:00	.94	.94	.69	136.2	136.2

series already described, the fourth consisted of plants in which the leaves were immersed in distilled water, just as the controls, except that the stems were also cooled. The results of these experiments are shown in figure 10.

Most of the preliminary experiments were performed by Mrs. R. Engelsberg. It took a considerable amount of work to establish the conditions under which the bleeding was controlled by the sugar content of the roots, so that applied sugar would increase the rate of bleeding. At first the results were irregular, and only about half of the experiments gave positive sugar effects. By narrowing down the optimal conditions, it became possible to get positive results in close to 100 per cent. of the experiments. Some

results obtained in these earlier experiments are presented in figures 4 and 5. When it was decided that the results were sufficiently reliable, the main series of experiments was started. Then all experiments were included in the calculations, so that the tables and curves give a complete picture of the effects obtained.

TABLE II

VARIATION IN THE BLEEDING RATE OF INDIVIDUAL PLANTS WITH STEMS AT 1° C AND CONTROL PLANTS

PLANT NO.	BLEEDING RATE, DROPS PER 3-HOUR PERIOD,* AS SHOWN BELOW			BLEEDING RATE EXPRESSED AS PERCENTAGE OF FIRST PERIOD		
	17:00–19:00	22:00–24:00	1:00–3:00	17:00–19:00	22:00–24:00	1:00–3:00
	STEMS AT 1° C, LEAVES IN 7% SUCROSE					
1	12.7	6.9	6.5	100	54.3	51.2
2	9.8	4.9	4.7	"	50.0	48.0
3	8.6	2.4	2.2	"	27.9	25.6
4	7.7	3.0	3.9	"	39.0	50.7
5	4.0	1.5	1.7	"	37.5	42.5
6	5.4	1.8	2.3	"	33.4	42.6
7	3.8	1.0	1.4	"	26.3	36.9
8	7.8	2.7	3.1	"	34.6	39.8
9	9.5	3.2	3.0	"	33.7	31.6
10	11.7	4.2	4.2	"	35.9	35.9
11	17.3	9.1	9.7	"	52.6	56.1
			Total	1100	425.2	460.9
			Mean	100	38.6	41.8
			Stan. Error		2.9	2.8
	STEMS AT 24° C, LEAVES IN WATER					
1	11.5	2.6	2.5	100	22.6	21.7
2	12.8	5.6	3.6	"	43.7	28.1
3	13.1	4.6	2.5	"	35.1	19.1
4	8.6	2.1	.9	"	24.4	10.5
5	16.1	3.7	2.4	"	23.0	14.9
6	16.4	5.1	3.8	"	31.1	23.2
7	5.8	.7	.3	"	12.1	5.2
			Total	700	192.0	122.7
			Mean	100	27.4	17.5
			Stan. Error		3.9	3.0
Standard Error of the Difference Between Means:					11.2 ± 5.06	24.3 ± 4.49
Odds that Such a Difference is not due to Chance:					36 to 1	100,000,000 to 1

* Period 17:00–19:00 actually includes 16:30–19:30, etc.

Results

In considering only the control plants, in which the leaves were submerged in water, there was found to be no great difference in the bleeding curves of two series—one in which the stems were cooled to 1° C or 5° C and the other with the stems at room temperature (23° C). If these two values are plotted as a percentage curve (bleeding of cooled plants over bleeding of normal plants) no sharp inflection is found as in the case of the sucrose-fed plants.

Tabulated results and the statistical treatment of three experiments, in which the stems were cooled to 1° C, are shown in tables I, II and III. Other temperatures used were tabulated in the same way but are not reproduced. However, all of the graphs prepared from the tables are shown, covering the complete range of temperatures used, as it is somewhat more convenient to visualize the results of the experiments from these. Although the recording of bleeding was started at 11:00 as indicated in table I and

TABLE III

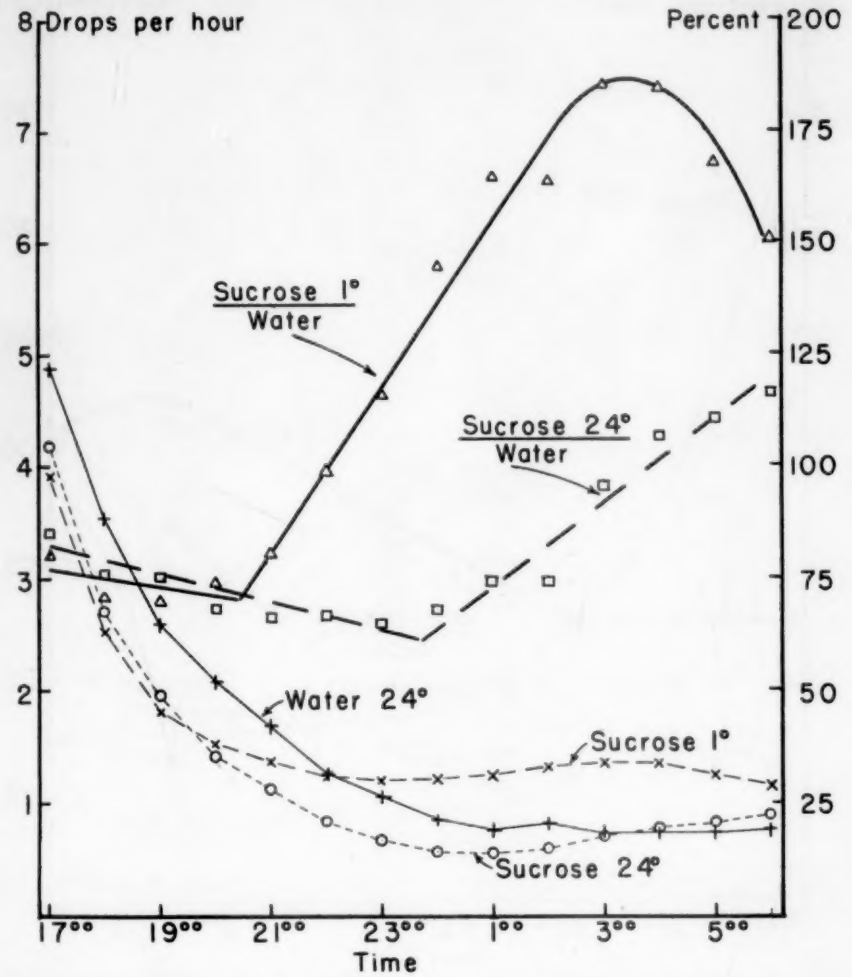
VARIATION IN THE BLEEDING RATE OF INDIVIDUAL PLANTS WITH STEMS AT 24° C AND CONTROL PLANTS

PLANT NO.	BLEEDING RATE, DROPS PER 3-HOUR PERIOD, AS SHOWN BELOW			BLEEDING RATE EXPRESSED AS PERCENTAGE OF FIRST PERIOD		
	20:00–22:00	1:00–3:00	4:00–6:00	20:00–22:00	1:00–3:00	4:00–6:00
	STEMS AT 24° C, LEAVES IN 7% SUCROSE					
1	2.9	1.4	1.8	100	48.2	62.1
2	5.4	3.3	6.1	"	61.2	112.8
3	7.1	1.6	1.9	"	22.5	26.8
4	5.2	1.9	2.0	"	36.6	38.5
5	1.1	.6	1.3	"	54.6	118.1
6	3.1	1.6	1.8	"	51.6	58.2
7	5.2	1.7	1.9	"	32.7	36.6
8	.5	.3	.5	"	60.0	100.0
9	3.9	3.2	4.9	"	82.1	125.6
			Total	900	449.5	678.7
			Mean	100	49.9	75.4
			Stan. Error		5.9	12.9
	STEMS AT 24° C, LEAVES IN WATER					
1	5.2	2.5	4.1	100	48.1	78.9
2	7.4	3.6	2.9	"	48.7	39.2
3	6.9	2.5	1.8	"	36.3	26.1
4	3.3	.9	1.1	"	27.3	33.3
5	6.0	2.4	2.4	"	40.0	40.0
6	7.6	3.8	3.1	"	50.0	40.8
7	2.0	.3	.3	"	15.0	15.0
			Total	700	265.4	273.3
			Mean	100	37.9	39.0
			Stan. Error		4.9	7.5
Standard Error of the Difference Between Means:					12.0 ± 8.57	36.4 ± 17.34
Odds that Such a Difference is not due to Chance:					5.2 to 1	27.0 to 1

the sugar was applied at 11:55, the graphical representation in figures 6, 7, 8, 9, and 10 begins at 17:00. It is thus possible to use a more convenient scale for the ordinate, as the very high values found during the first several hours of bleeding (due to watering the plants) need not be included. All significant changes occur during the time interval shown in the graphs.

There are large differences in the rate of bleeding of the individual plants. Therefore a statistical analysis of the rates of bleeding of sugar-treated and non-treated plants will not show significant differences. The essential effect

which was measured, however, was the change in rate of bleeding of the treated when compared with control plants. Therefore the rates before and after sugar had taken effect were compared as follows: For each individual plant the basic bleeding rate for the period just preceding the onset of the sugar effect, as evidenced by the point of inflection on the percentage curve, is taken as 100 per cent. Then, after the applied sugar has started to take



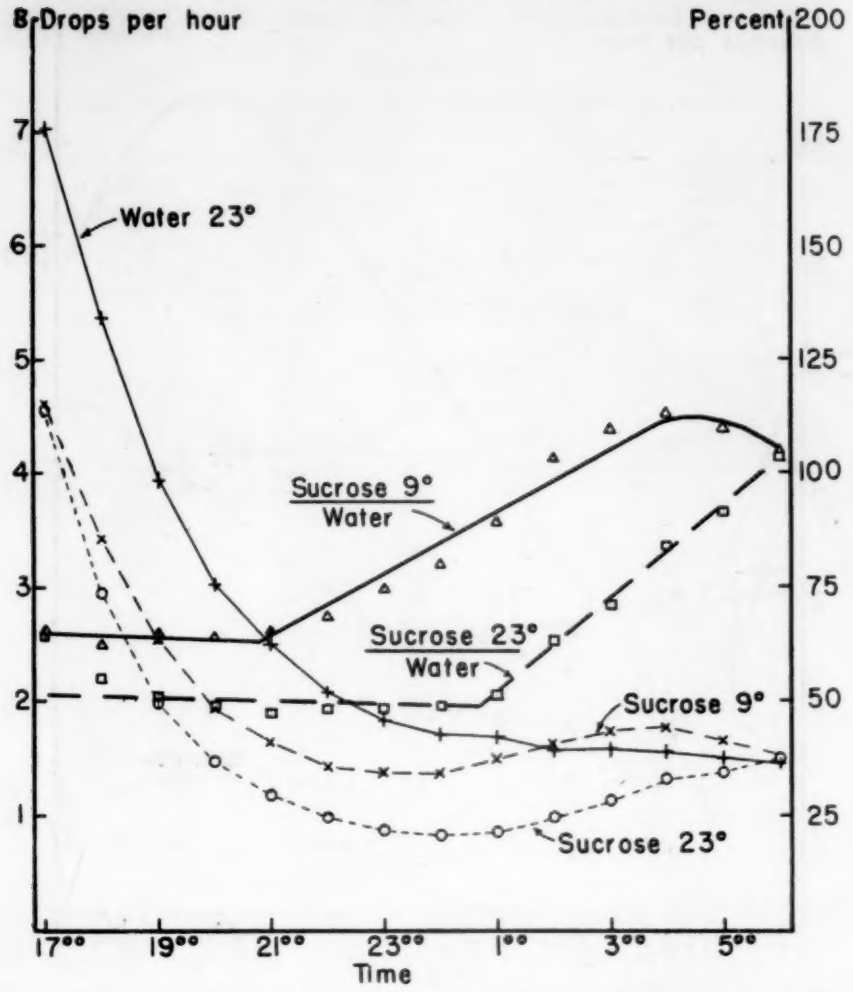
effect, the bleeding rate is recorded for two three-hour periods, as percentage of the basic rate. This same calculation is made for the control plants for the same time intervals.

The individual variability is then calculated from the percentage figures, and the statistical significance of the sugar effect can be judged from the standard error of the difference between sugar-treated and control plants.

Table II shows these data for the plants of which the stems were chilled

to 1° C. The differences in the rates of bleeding for the individual plants were very high. But when expressed as percentage of the basic rate from 17:00 to 19:00, the variability was rather small. In table III the same calculations were made for the plants of which the stems were kept at room temperature.

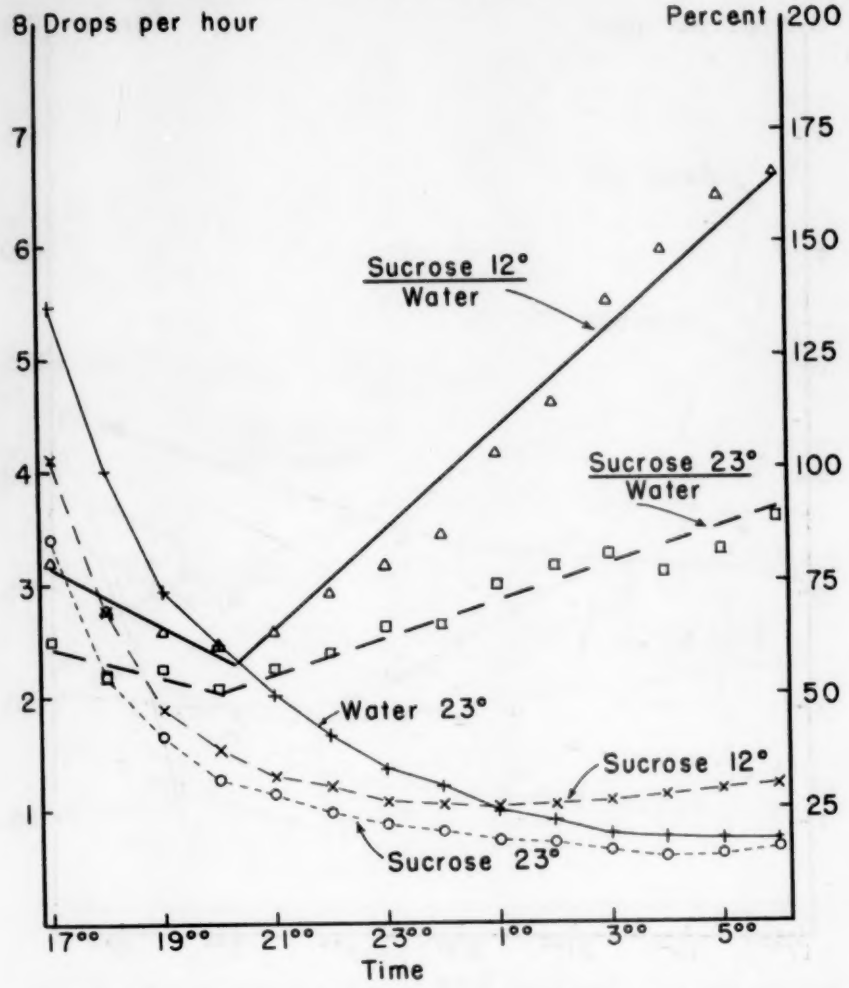
If, for the percentage values, the standard error of the differences be-



tween the means of the cooled, sucrose-fed plants, and the corresponding controls is examined, it is seen at once that the difference is highly significant. The sucrose-fed plants kept at 24° C compared to their corresponding controls have a somewhat higher standard error, but the difference is still fairly significant. The odds are shown in tables II and III.

Of the more than 400 plants of which the bleeding was recorded, less than a dozen behaved contrary to what was found in all others. They, however,

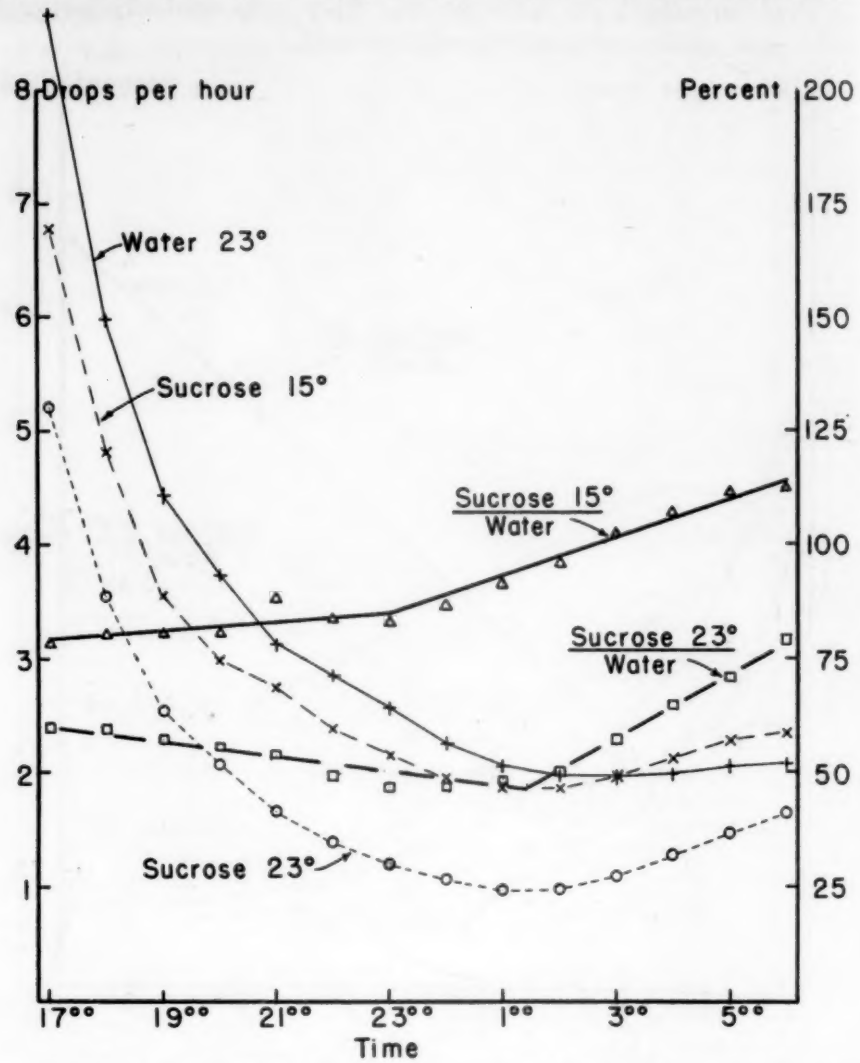
were averaged with the rest of the plants. Such abnormalities appeared to occur more in the late summer when both the rate and the regularity of bleeding were not so high as during the early and midsummer. The only plants not included in the averages were the few which did not continue to bleed throughout the entire period. They were randomly scattered throughout all the series at all temperatures used.



The five graphs representing all experiments (figs. 6–10) are presented as a group as they were all prepared in exactly the same way. Tables I, II and III refer only to the first of these graphs (fig. 6). The graph for the 5° C experiments is placed last (fig. 10), as it contains the extra series of plants previously described.

The first thing that is apparent in the graphs is the earlier upward inflection of the percentage curves in the case of cooled plants as compared with

those at room temperature (23° ± 1° C). The only exception is figure 8 where the inflection for the 12° C plants and the 23° C plants both occur almost simultaneously, at 20:10. Sucrose was applied to the leaves at 11:55, and if the graphs are examined, it may be seen that the time interval be-



FIGS. 6–9. Rate of bleeding of plants having two leaves in water or two leaves in 7 per cent. sucrose. Stems of one group of sugar-treated plants cooled to 1° C (fig. 6), 9° (fig. 7), 12° (fig. 8) and 15° (fig. 9). In each figure the proportion of the rate of bleeding of both groups of sugar-fed plants in per cent. of that of the water controls is plotted.

tween application and an increased metabolic activity in the roots, as shown by an upward inflection in the percentage curves, ranges from 8.2 hours to

13.4 hours. This is shown in figure 11. The point at 23° C is obtained by averaging the control plants of all five series. There appears to be a fair degree of correlation between temperature of stem and rate of translocation.

Analysis of sucrose content of the roots showed rather small values, ranging from 2.04 to 8.30 mg. sucrose per gram of dry tissue. They are tabulated

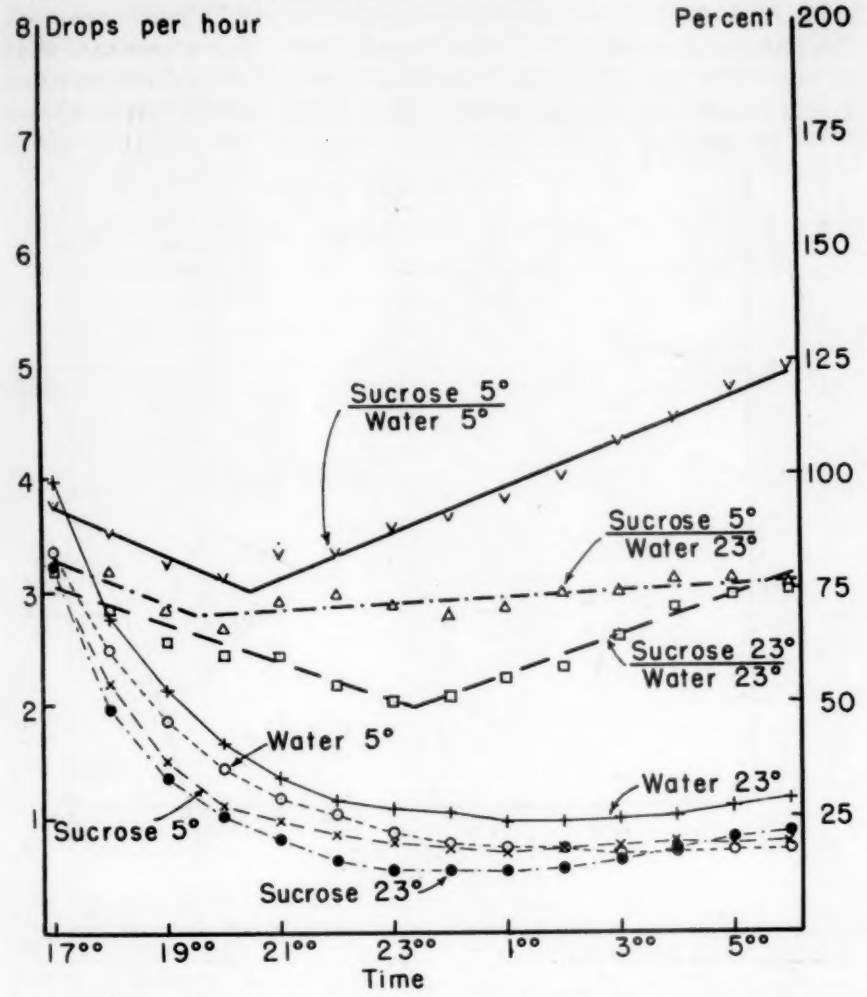


FIG. 10. Same as figures 6–9, but in addition the bleeding of control plants with leaves in water, and cooled stems is plotted.

in table IV. There appeared to be no correlation between control plants and sucrose-fed plants (regardless of the stem temperature), as far as sucrose content was concerned. However, in considering only the plants which were fed sucrose, it was found that in 16 out of the 17 experiments which are indicated in this work, a higher content of sucrose existed in roots of plants which had their stems kept at room temperature. The average difference

between these two series of plants in the 16 experiments amounted to 1.6 mg. of sucrose.

Discussion

The experiments described in the experimental part, although showing a fairly large amount of individual variation, indicate that as a result of application of sucrose to tomato leaves, approximately 10 hours later a sudden increase in activity of the root system occurs. This is measured as an increase in the rate of bleeding from the stump of the stem above the leaves. This is actually due to transport through the living tissues of the stem, since

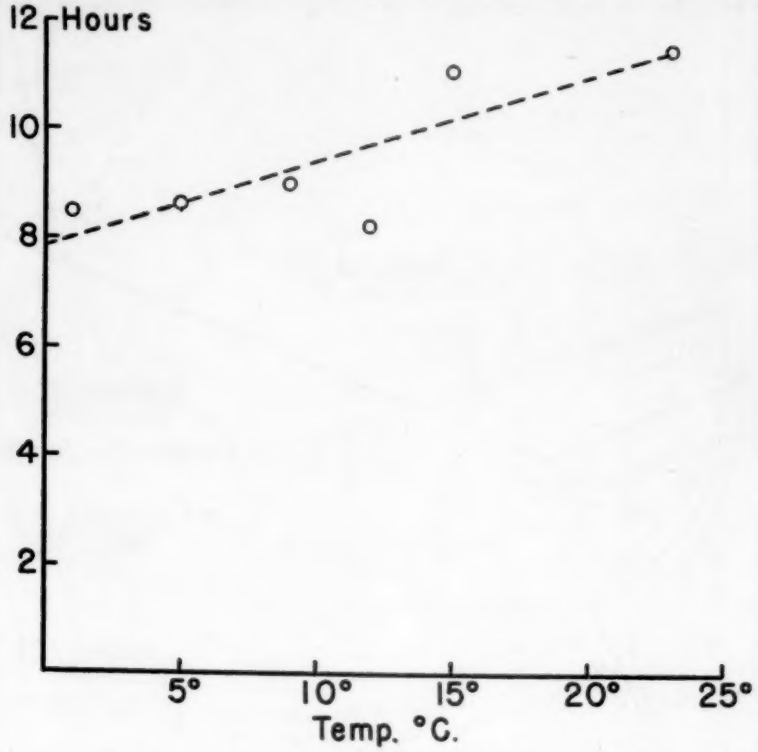


FIG. 11. Relationship between temperature of the stems of the sugar-fed plants and the length of time it takes before the effect of the applied sugar can be observed (plot includes all experiments).

the sugar effect on bleeding is absent when a short section of the stem is killed with steam, and is greatly reduced when wounds have been made along the path of translocation through the stem. Such wounds do not interfere with the basic rate of bleeding, they only affect the sugar effect (WENT and CARTER, **19**). This also proves that the increased rate of bleeding has its focus of activity below the wound, and not in the upper parts of the stem. During the first 10 hours after application of sugar the plants whose leaves are submerged in water, bleed consistently one to two drops per hour more

than the sugar-treated plants. This is due to the osmotic effect of the 7 per cent. sugar solution. Whereas the tubes containing water lose 3.6 cc of water towards the immersed leaves, the tubes containing 7 per cent. sucrose lose only 0.8 cc during the same period. Notwithstanding this additional amount of bleeding sap to be moved upward through the stem, the absolute amount of bleeding sap expressed through the cut surface 17 hours after beginning of the sugar treatment, exceeds that of the control plants in all cases when the stems are cooled to 1–15° C, and in two out of five cases at room temperature.

In four out of five groups of experiments the increase in the rate of bleeding due to sugar application starts earlier when the stems are cooled

TABLE IV

SUCROSE CONTENT IN MG. PER GRAM DRY WEIGHT OF TOMATO ROOTS AT THE CONCLUSION OF THE BLEEDING EXPERIMENTS, AND THE RATE OF BLEEDING DURING THE HOUR PRECEDING THE HARVEST OF THE ROOTS FOR SUCROSE ANALYSIS

TEMPERATURE OF COOLED STEMS	EXPERIMENT NUMBER	LEAVES IN SUGAR, STEMS COOLED	LEAVES IN SUGAR, STEMS AT 23°–24°	LEAVES IN WATER, STEMS AT 23°–24°
	17	5.50	7.70	7.70
0–1°	18	3.76	4.30	3.54
	20	4.08	5.26	4.94
	19	3.97	4.50	4.61
8–9°	21	3.89	4.10	4.63
	22	2.84	7.68	4.10
	28	4.50	4.65	2.85
	25	3.37	5.27	.....
12°	26	3.07	4.27	4.60
	23	2.74	6.84	4.00
15°	24	4.52	7.16	6.00
	27	4.50	7.00	3.84
	31	4.85	5.46	5.22
	32	5.70	6.52	5.34
5°	34	6.20	8.30	5.27
	35	6.20	8.30	4.35
	36	4.72	4.10	2.04
Mean sugar 0/00		4.38	5.97	4.56
Bleeding (drops/hour/plant) prior to sugar sampling		1.11	1.04	0.88

to 1–15° C, than when they are kept at 23–24° C, and in all cases the cooled plants bleed more profusely than the control plants. This means not only that the applied sugar takes effect in the root system earlier, but that translocation is enhanced at the lower temperatures. The latter is in complete accordance with the earlier observations and measurements on the tomato plant (WENT **17**). Furthermore, it should be stated that the same was observed by Mrs. Engelsberg in her experiments carried out in the spring of 1946. Since her records are not as extensive as those presented in this paper, they will not be reported, but when the stems were kept at 5°, 6° and 9° C, she also found a more rapid and more extensive bleeding than at room temperature, when sugar was applied. This means that the observed acceleration and intensification of sugar transport through tomato stems at lower

temperatures is reproducible and can be accepted as an established fact. The calculations of tables II and III also prove that the effect of sugar application is statistically significant.

Figure 11 shows a fairly good correlation between the temperature at which the stems were kept and the rate of sugar transport. It has to be borne in mind that this curve represents the length of time required for the sugar to be taken up through the leaves, to enter the sieve tubes, to move a distance of approximately 40 cm. through petiole, stem and root, and to become active in the root system. Only 20 cm. of the distance travelled in the sieve tubes is kept at the lower temperatures so that the three-hour time difference between transport at 1° and 23° C represents only half of the actual length of transport. Therefore the time required for the transport from leaf to root at 23° is at least six hours, even if transport at 1° is considered to require no time. We can say then, that the time required for sugar entrance into leaf and sieve tubes, transport through the sieve tubes at 1° C, and its becoming effective in the roots, is five and one-half hours. This also explains why these bleeding experiments have to be carried out in a cool atmosphere, and why experiments in a room kept at 27° C gave consistently negative results as far as effects of applied sugar were concerned.

It is possible to plot not only the rate of sugar transport, but also the relative amount transported can be judged. As discussed earlier, the sugar-fed plants exude less than the water controls, because of the osmotic pull of the sugar solution. If this osmotic effect is eliminated by extending the bleeding curves for the sugar-fed plants parallel with the water controls, the area between this hypothetical curve and the real curve indicates the number of drops of bleeding sap which were exuded as a result of the sugar treatment. This is shown in figure 12, which indicates that somewhere near 30° C transport through the stem in the San Jose Canner is reduced to practically zero. This corresponds well with figure 17 from WENT (**17**), in which also 30° is the critical value, above which it was inferred that no sugar transport towards the roots could occur. Since in the experiments carried out at 5° the only six plants occurred, which did not show an increase in bleeding in response to sugar application, the mean values for the sugar effect are necessarily low, and therefore this series of experiments was not plotted in figure 11.

The sugar analysis on the roots at the conclusion of each experiment show an apparent negative correlation with the bleeding data. However, in all cases the roots were harvested several hours after the maximum sugar effect had occurred. In several instances the bleeding of the sugar-fed plants with cooled stems by that time had dropped again below that of the water controls in five out of 15 cases or below that of the sugar-fed plants kept at room temperature (in seven out of 15 cases) (see *e.g.* figures 6 and 7). Therefore the sugar analysis was not carried out when the differences in rate of bleeding were most pronounced, but when these differences had largely

disappeared or even were reversed. This invalidates a comparison between sugar content of roots (table IV) and the sugar effects on bleeding as shown in figure 12, and there is no discrepancy between these data. Therefore, too, it is unnecessary to assume that the sucrose was not translocated as such, but as a derivative which caused the increased rate of bleeding. Regardless of the explanation of this phenomenon, the results of this investigation prove that the physiological effect of applied sugar on bleeding is exerted by the intermediary of a translocation process, which occurs faster and more intensely at lower temperatures.

The fact that, at least in the tomato under the conditions of the experiments, the sugar transport is accelerated by lowering the temperature,

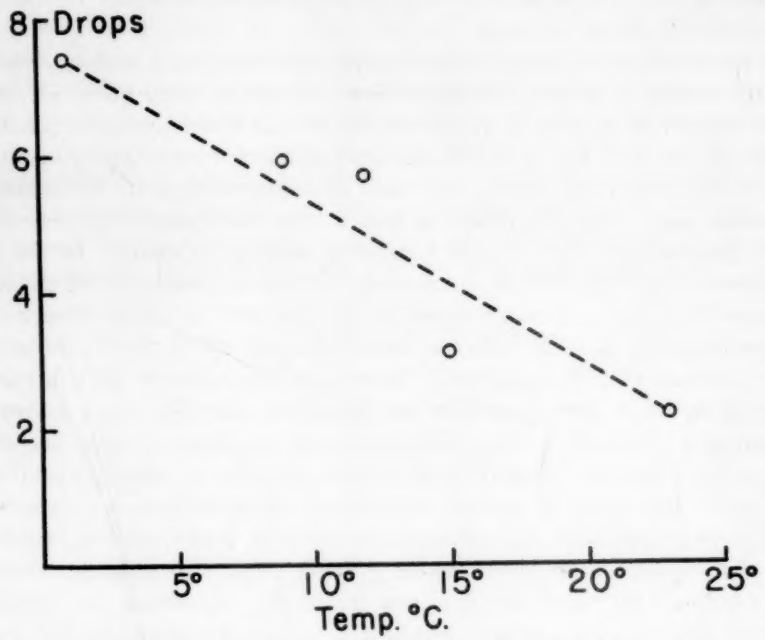


FIG. 12. The amount of increased bleeding due to applied sugar as a function of the temperature at which the stems are maintained.

has a number of interesting corollaries. It makes it very hard to assume, that the motive force for sugar transport is located in the chilled part, for most life processes are slowed down very much at temperatures near freezing. It excludes, for instance, that protoplasmic streaming is the agent causing sugar movement through the phloem. The schemes which suggest movement along interfaces, or accelerated diffusion, could be expected to have also a Q_{10} of 1 or slightly above 1. Therefore, we must conclude that the movement through the phloem is passive, even though the sieve tubes have to be alive to permit passage of the sugar. This suggests that the only acceptable existing theory of sugar transport in the tomato

plant is that of Münch. But this theory does not predict a Q_{10} below 1, so that we have to find a reason why mass flow of a sugar solution would be facilitated by low temperature.

There are several possible explanations of how a physiological process can have a Q_{10} of less than 1. We know that the Q_{10} of respiration, growth, etc., is less than 1 above the optimal temperature. In many such cases this is due to an injury-reaction. Such an injury reaction has a Q_{10} which is much higher than the Q_{10} of the process which is injured, and thus an over-all Q_{10} of less than 1 results. Such injury occurs only at temperatures above 30° C, where coagulation of proteins and inactivation of enzymes set in. In the translocation of sugar, however, the low Q_{10} holds even for temperatures close to freezing, where no injury due to high temperatures can be expected.

We also might assume that there exist reactions with a high Q_{10} inhibiting in one way or another the sugar flow. Since in many cases the capacity of sugar transport is limiting the growth and development of plants, it is hard to see why the plant should have developed extra obstacles in the way of this sugar transport. It could be expected that in the course of evolution such inhibiting reactions would have disappeared through selection. Therefore it seems more logical to look for a change in the flow resistance with temperature. Actually there is a possibility of bringing the mass flow theory of sugar translocation in accord with our observations.

According to MÜNCH (**10**) the sugar solution which flows through the phloem remains in the symplast. WENT and ENGELSBERG (**21**) have suggested that the sucrose formed by photosynthesis actually never leaves the protoplasm. The sieve tubes through which the sugar moves are filled with a very thin protoplasmic mass, which seems to be equally distributed throughout the cells. Thus this protoplasm will constitute a resistance to the flow of sugar solution between its interstices, proportionate to the degree of swelling of the protoplasmic colloids. Since increase in temperature results in increased swelling (DE HAAN, **6**), decreasing the total free path for solution, this concept would account for decreased flow of sugar solution at higher temperatures. This view is not necessarily in conflict with the views expressed by DE HAAN (**6**) who attributes increased permeability for water to swelling of the protoplasmic colloids. But in his case he considers only the protoplasmic lining of cells, where swelling of the protoplasmic colloids means increase in pore diameter between micells, when through swelling these micells are forced apart. In cells completely filled with protoplasm the micells cannot be forced apart, but swelling means decreased interstitial space.

We can therefore conclude, that the reported experiments do not support the activated diffusion or protoplasmic streaming theories of sugar translocation, but can be interpreted provisionally on the basis of the Münch theory of mass-flow.

The method employed to measure the rate of translocation of sugar can

be used for the determination of other substances as well, as long as they affect the activity of the root system. By making auxin or vitamin B_1 the limiting factors for bleeding, instead of sugar, the rate of bleeding would change upon their application.

Summary

Tomato plants with healthy root systems will bleed for a considerable time after decapitation. The rate of bleeding depends upon the activity of the root system, and this in turn is affected by its sugar content. By recording the rate of bleeding any changes due to sugar applied to the leaves could be measured. It was found that when 7 per cent. sucrose was applied to two leaves, inserted about 25 cm. high on the stem, the rate of bleeding suddenly increased about 11½ hours after immersion of the leaves in sugar. By varying the temperature along a 20 cm. length of stem the effect of temperature on the rate and intensity of sugar translocation could be measured in terms of bleeding. It was found that the time necessary for the sugar to take effect decreased as the temperature of the intermediate stem piece was decreased (fig. 11). The amount of sugar translocated gradually increased as the temperature was lowered (fig. 12). This means that in the tomato plant both the rate and intensity of sugar transport have a Q_{10} well below 1.

KERCKHOFF LABORATORIES OF BIOLOGY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA 4, CALIFORNIA

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BRIEF PAPERS

MOVEMENT OF P^{32} IN MATURING CORN PLANTS

CARL L. WITHNER[1]

(WITH TWO FIGURES)

Received November 12, 1948

As the result of an experiment to produce radioactive calcium phytate (**3**) some extra corn plants and radioactive phosphorus were available for other use. It was decided to study the movement of the P^{32} in the maturing plants. BREWER and BRAMLEY (**1**) have studied the uptake of radioactive phosphorus in corn seedlings, and Hevesy and his coworkers (**2**) have noted the concentration of isotopic phosphorus in corn leaves of different ages. No data were available, however, on the translocation or accumulation of this isotope in mature corn plants with ripening ears.

Methods

The corn used in the experiment was Connecticut 520, and it was grown in the greenhouse in gravel culture, according to the procedure described

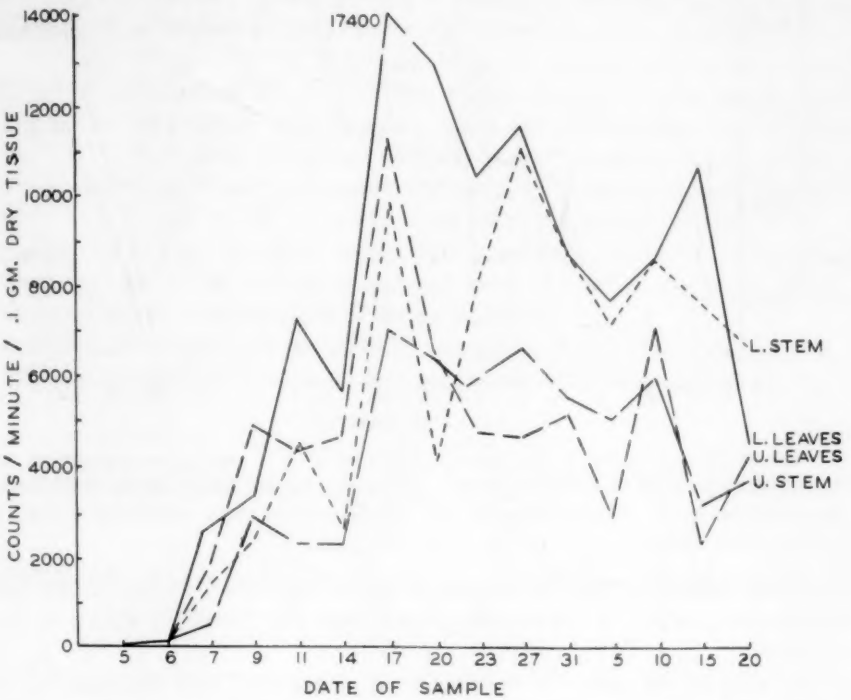

FIG. 1. Graph showing the concentrations of P^{32} in successive samples of the foliage parts of the corn plants.

[1] Present address: Biology Department, Brooklyn College, Brooklyn 10, New York.

by JACOBSON (**3**). The introduction of the radioactive phosphorus, which was in KH_2PO_4, to the nutrient solution is similarly described by him.

For this experiment the radioactive solution was put on the corn at 5 P.M. of May 5. The first sample was made at midnight, the next the following noon, and succeeding ones at intervals gradually increased to five days. The later samples were all made at noon. When the P^{32} was first applied on May 5 the plants were in the early milk stage. They had started to tassel on April 15, and the first silk appeared on April 25. The ears were ripe by June 10, and the experiment was continued ten more days until the plants were beginning to dry.

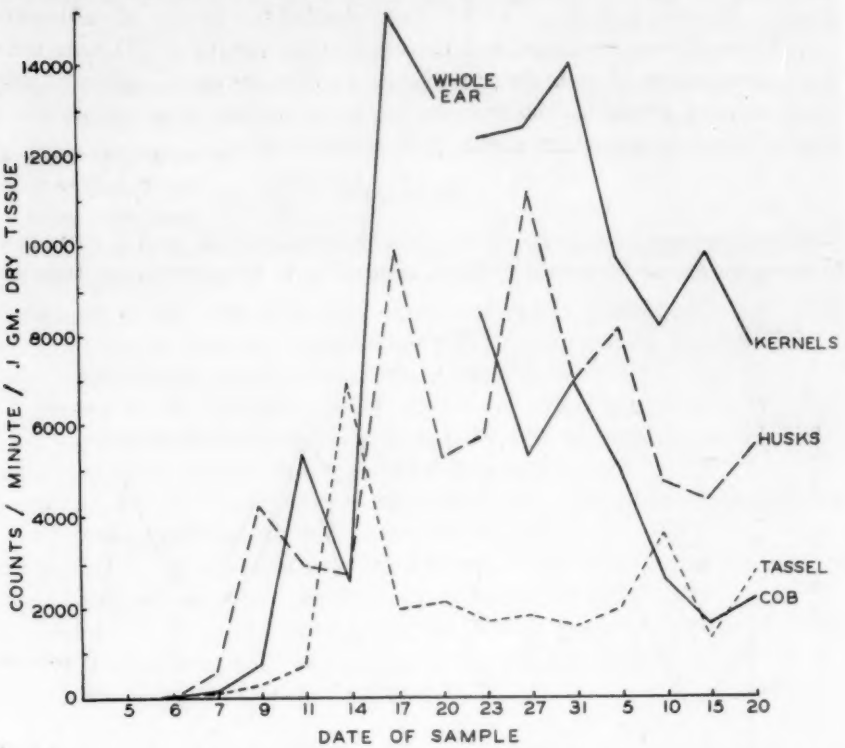

FIG. 2. Graph showing the concentrations of P^{32} in successive samples of the reproductive portions of the corn plants. The curve for the whole ear is continued as two separate curves, kernels and cob, from the time these parts were large enough to separate conveniently.

After each plant was harvested, it was cut up, dried, and ground. The radioactivity of a .1 gram sample of each part was measured with a Geiger counter.

Because of the limited number of corn plants (15) only one plant could be harvested for each group of samples. This undoubtedly produced discrepancies because it was impossible to keep selecting the plants so they would come in exact order as regards their stage of development. There-

fore, the curves fluctuate more widely than they should, as the samples were not always of progressive morphological age. For this reason the significance of any changes in concentration aside from general trends should not be taken too seriously. The overall picture is of interest, however, to show the speed of uptake and general distribution and translocation.

The division of the plants into upper and lower stems and leaves was made at the node at which the first ear was attached. The ear was separated into kernels and cob as soon as it was large enough to make this practicable.

Results and discussion

The results are primarily expressed in the form of two graphs (fig. 1 and 2). It can be seen that the radioactive phosphorus increased rapidly in concentration in the plants until a maximum was reached after twelve days. The concentration of the P^{32} in each of the foliage parts then gradually decreased to approximately the same value. The lower stems and leaves contained more P^{32} than those above the ear, and the leaves contained more than the stems. The concentrations of phosphorus in the lower leaves and stems paralleled the concentrations in the upper parts of the plants. The radioactive phosphorus in the tassel increased slowly and the concentration remained about constant afterward.

Husks contained more P^{32} than the cob, and the concentration increased to a maximum, then declined. P^{32} was almost completely translocated from the cob by the time the ear was ripe. The kernels contained the greatest concentrations of this phosphorus of all tissues at the end of the experiment. The 83 grams of kernels (47 grams dry weight) showed approximately the same total activity as 265 grams of lower stem (56 grams dry weight).

The general movement of the phosphorus can be observed by a peak of activity in the stems before one in the leaves. Likewise, the husks show a peak before the one in the ear or tassel.

When the leaves and stems were beginning to dry after the ears had matured, a residual amount of activity remained in the cells of all plant parts.

Osborn Botanical Laboratory
Yale University
New Haven, Connecticut

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FLOWER INITIATION IN TOTAL DARKNESS

A. Carl Leopold

(with two figures)

Received December 1, 1948

A large number of experiments have been reported in the literature on photoperiodism indicating that light is essential to the photoperiodic induction of flowering. For example, Klebs (**5**) was unable to find flower primordia in darkened Sempervivum, Garner and Allard (**3**) found that prolonged darkness would not produce flowers in long- or short-day plants, Borthwick and Parker (**2**), Hamner (**4**) and Mann (**7**), have found minimal daily light periods below which flower initiation would not take place in several species. These observations were made with initially green, leafy plants.

On the other hand, it is curious that there are several cases of etiolated plants which have never experienced normal daylight intensities, but which are capable of initiating flowers. A notable case is that reported by Spoehr (**9**), who grew corn plants by a sugar feeding technique from germination to fruition in the near absence of light (½ hour of weak light each day). Spoehr obtained similar results with sunflower as well (personal communication). Another case is reported by Reed and MacDougal (**8**) who found that the saprophyte Corallorhiza is able to initiate flowers in complete darkness. Also it seems likely that the same is true of other saprophytes such as Monotropa which develop vegetatively entirely underground, presumably in the absence of light, and there initiate flowers which subsequently push up out of the ground. Borgström (**1**) has reported that etiolated American Wonder peas developed flowers and even fruits in complete darkness.

It appears at least possible that truly etiolated plants may not require light for flower initiation. To test this possibility several species were planted in a constant temperature darkroom (25° C.) and raised in the complete absence of any light from the time of planting.

Biloxi soybeans failed to produce recognizable flower primordia in the dark.

Irish Cobbler potatoes, grown from eight intact tubers, had developed abundant flowers on every plant after six weeks in the dark. The first fifteen nodes of each plant lacked flower primordia in every instance, but nodes above that produced abundant visible flowers (fig. 1)—sometimes more than twenty flowers at a node. In one particularly large plant, flowers were so profuse that there were no leaves produced at all for as many as five consecutive nodes. The flowers developed no corollas, and were never observed to open. Control plants grown in the greenhouse under short days (10 hours) were still entirely vegetative after the six-week

FIG. 1. Unopened flowers of potato produced on etiolated plants in the complete absence of light (×5).

period. Plants which were moved from the short-day to a long-day treatment (16 hours) developed flower buds after 10 days.

FIG. 2. Spherical flower primordia of pea produced on etiolated plants in the complete absence of light (×30).

Alaska peas developed flower primordia at the seventh node 18 days after germination in the dark (fig. 2). Plants grown in the greenhouse in a long day (16 hours) developed flower primordia at the seventh node also, but plants grown in a short day (10 hours) were entirely vegetative after several weeks in which at least twelve nodes were developed.

Red kidney beans developed flower primordia in the dark at many nodes, from the cotyledon axils and the seed leaf axils on up. Plants grown in the greenhouse produced flower primordia at about the same places.

The fact that these three species will initiate flowers in complete darkness raises the following questions:

1) Is light necessary for the formation of the hypothetical flowering hormone? If the hormone cannot accumulate in the dark one would hardly expect the etiolated potato plants to be vegetative for the first 15 nodes and then initiate flowers in profusion. When an abundance of stored materials is available in the form of a potato tuber or pea or bean cotyledons, it appears that the plant may be capable of forming the flower-inducing substance in darkness.
2) Why will not continuous darkness permit flower initiation in green plants? If some etiolated plants will flower in complete darkness and green plants will not when placed in complete darkness, there must be some inhibition introduced by the green leafy condition. It seems likely that the photoperiodic requirement is a means of overcoming this inhibition. The "secundären Hemmungsprozesse" of LANG and MELCHERS (**6**) may be just such a process.

Grateful acknowledgment is given to Professor K. V. Thimann for his suggestions and criticisms.

HAWAIIAN PINEAPPLE COMPANY
HONOLULU, HAWAII

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THE EFFECT OF TEMPERATURE ON THE SUSCEPTIBILITY OF PLANTS TO 2,4-D

SALLY KELLY

(WITH TWO FIGURES)

Received November 26, 1948

The conditions under which 2,4-D is most effective as a weed-killer are not thoroughly established. Among the possible factors influencing the result of 2,4-D application is the temperature at which the plants are growing. HAMNER and TUKEY (**2**) noticed that killing of bindweed by 2,4-D was more rapid in midsummer than in the fall. MARTH and DAVIS (**3**) found that 2,4-D killed lawn weeds at a more rapid rate when they were growing at warmer temperatures. Both the absorption and translocation of 2,4-D in bean plants were speeded when they were kept at higher temperatures (**4**). Akamine's recent experiments indicate that root induction by 2,4-D is influenced by temperature (**1**). The following experiments were carried out to investigate more precisely the relationship between temperature and susceptibility of plants to 2,4-D.

Methods

Greenhouse-grown plants of red kidney bean, perennial rye and crabgrass, three species which vary in their reactions to the weed-killer, were sprayed with aqueous solutions (plus Carbowax as a wetting agent) of 2,4-dichlorophenoxyacetic acid (potassium salt) and placed in chambers of constant temperature and equal light intensities. The plants were sprayed with concentrations from 0.001 to 0.5 per cent., depending upon the species.

Results

The three species of plants were more susceptible to 2,4-D at higher temperatures than at lower ones, a characteristic pointed out in previous investigations (**2**) (**3**). In bean (Fig. 1), this difference in susceptibility was observed with concentrations of from 0.001 to 0.5 per cent. 2,4-D and, when 0.05 per cent. was used, was apparent two days after spraying. Table I lists the minimum concentrations tested that brought about killing of bean at the temperatures used.

Preceding the killing process, bean plants exhibited growth responses that also varied according to the temperature at which they were placed after spraying. Bending of young stems, twisting of petioles, and leaf curling were more pronounced at 25° C. than at lower temperatures. Perennial rye and crabgrass, however, exhibited no growth responses but were killed by 2,4-D spraying. In crabgrass, about 30–40 per cent. of the killing at 25° C was caused by the high temperature itself or by some secondary factor since the unsprayed control plants exhibited yellowing at that temperature.

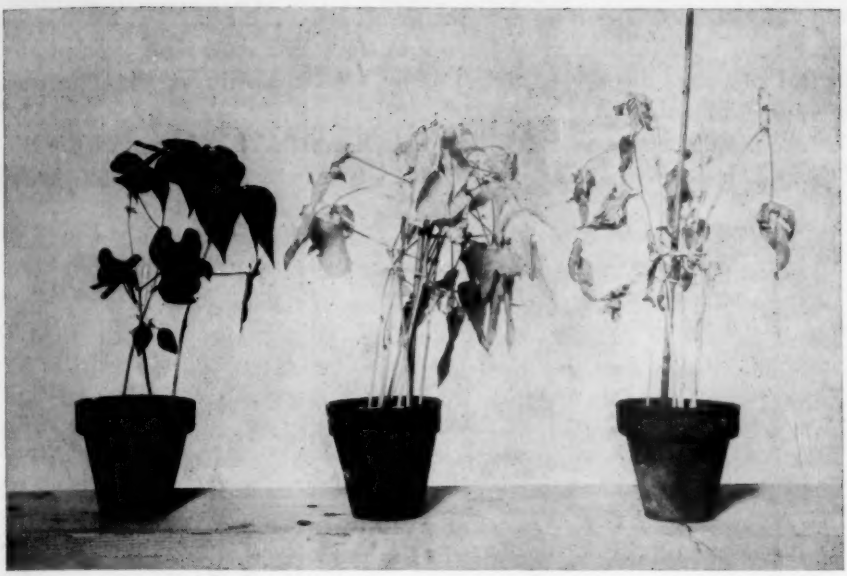

FIG. 1. Red kidney bean plants one week after they had been sprayed with 0.1 per cent. 2,4-D plus 0.5 per cent. Carbowax and placed at temperatures of (left to right) 5, 15, and 25° C.

2,4-D retained its activity during the period that plants were kept in the cold. When such plants were removed to higher temperatures after at least a week at lower ones, a procedure carried out by MARTH and DAVIS (**3**) with lawn weeds, the response to 2,4-D was increased (fig. 2). This could not be ascribed to additional penetration at higher temperatures since washing the leaves at the time of transfer (in an attempt to rid them of excess 2,4-D) did not decrease the subsequent response to 2,4-D. The washing process was carried out by directing a stream of water on the leaves and stems while they were rubbed gently between the fingers.

That the higher temperatures affect the sensitivity of the cells directly was shown in an experiment in which plants were placed at different temperatures (5, 15, and 25° C) for a week before spraying. When sprayed and then placed at a common temperature (15° C), plants previously kept at 25° C exhibited greater stem curvature, leaf curl and swelling than did those previously kept at 15 and 5° C. Previous holding of perennial rye grass at different temperatures resulted in greater killing when 15

TABLE I

MINIMUM CONCENTRATIONS OF 2,4-D THAT BROUGHT ABOUT KILLING OF RED KIDNEY BEAN PLANTS

TEMP. °C.	CONCENTRATION IN PER CENT
5	> 0.5
15	0.5
25	0.01

and 25° C were the pretreatment temperatures. The results of this experiment agree with those of other investigations which show that when physiological processes are slowed down, as by low temperatures, the plants are less susceptible to 2,4-D.

The author wishes to thank Professor Kenneth V. Thimann of the Biological Laboratories of Harvard University where this work was carried out for his hospitality and for his stimulating interest and advice throughout the course of the investigation.

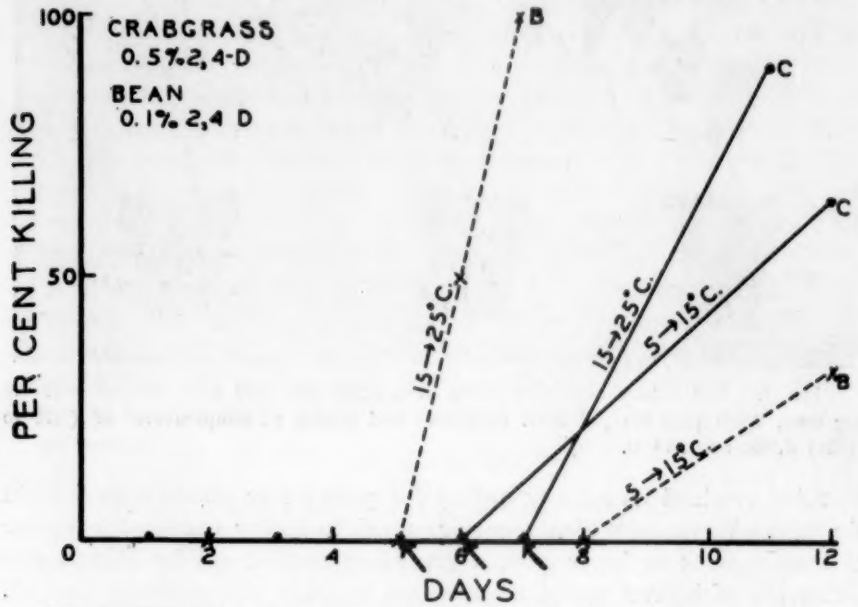

FIG. 2. Response of crabgrass and bean to 2,4-D after transfer from lower to higher temperatures. Arrows refer to the number of days after spraying that the transfer took place. All the bean plants were transferred five days after spraying. Solid line, crabgrass; broken line, bean.

VASSAR COLLEGE
POUGHKEEPSIE, NEW YORK

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A METHOD FOR THE STUDY OF FOLIAR ABSCISSION *IN VITRO*[1]

FREDRICK T. ADDICOTT, RUTH STOCKING LYNCH,
GEORGE A. LIVINGSTON[2], and JEAN K. HUNTER

(WITH EIGHT FIGURES)

Received December 27, 1948

During a series of experiments on abscission of leaves a method was devised which made possible the examination of this process in the laboratory under controlled conditions. Essentially, this method consists of excising small pieces of tissue including one or more abscission zones and using these pieces, called explants, for experimentation. Such explants have been made from Citrus (Valencia orange), Phaseolus (Black Valentine bean), and Coleus.

The Citrus explant includes ten millimeters of midrib and six millimeters of attached petiole (fig. 1). The most favorable bean explants are taken from trifoliolate leaves and are of two kinds: "a," the pulvinus and ten millimeters of the stalk of the terminal leaflet, and "b," the two pulvini of the lateral leaflets and ten millimeters of the attached proximal part of the leaf stalk (fig. 4). The Coleus explant consists of a node with the pair of attached petioles, each cut to six millimeters in length, and ten millimeters of the stem proximal to the node.

The presence of molds or bacteria on or near the explants can seriously affect a process as sensitive as abscission. Aseptic conditions are maintained during experiments by the use of sterilized solutions and glassware, by flaming instruments, and by setting up and examining the explants in a small transfer room that is sterilized by means of an ultraviolet lamp (Westinghouse Sterilamp). A number of disinfectants were tested for their ability to sterilize explants without injury to the tissues and without affecting the rate or incidence of abscission. These included bromine water, sodium hypochlorite, tergitol #7[3], roccal, zephiran chloride, lignasan, calcium dimethyldithiocarbamate, calcium ethylene-bisdithiocarbamate, sodium ethylene-bisdithiocarbamate, and phenylmercury oleate. None was fully satisfactory; roccal gave the best results. The optimum treatment was found to be a ten minute immersion in a 0.006 per cent. roccal followed by blotting.

[1] This paper is based upon work sponsored in part by the Biological Division, Chemical Corps, Camp Detrick, Frederick, Maryland, under Contract No. W-18-035-CM-208 with the University of California.

[2] Present address: Department of Biology, Reed College, Portland, Oregon.

[3] Sources of these reagents were as follows:
Tergitol manufactured by Carbide and Carbon Chemicals Corporation, New York;
Roccal obtained from Winthrop-Stearns, Inc., New York;
Ziphiran chloride manufactured by Winthrop Chemical Company, New York;
Various thiocarbamates and phenyl-mercury oleate obtained from Dr. W. H. Tisdale, Pest Control Research Station, E. I. duPont deNemours and Company, Wilmington, Delaware.

The explants were treated by mounting them on agar containing test substances, by immersion, by injection, by the application of small drops. Various methods of support were used. In the experiments with Citrus and Coleus and the early experiments with beans, the explants were mounted on 4 per cent. agar in Petri dishes. After the agar had set, an inch wide strip of it was removed along a diameter of the plate, leaving a narrow bridge of agar across one end to hold the agar in place and to serve as a marker. Ten explants were mounted on the agar of each dish, the shorter part of each explant (pulvinus or petiole) overhanging the central channel (figs. 2, 3).

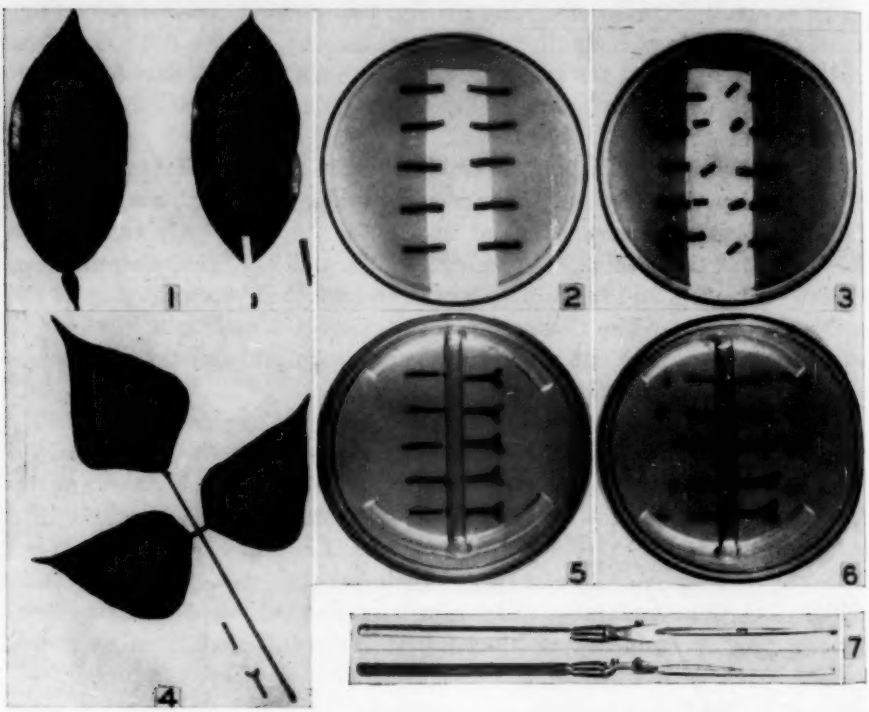

FIG. 1. Citrus leaves showing portion removed for an explant.
FIG. 2. Citrus explants on agar at the start of an experiment.
FIG. 3. Citrus explants on agar showing the effects of an experimental treatment.
FIG. 4. Bean trifoliolate leaf with explants.
FIG. 5. Bean explants on holders at the start of an experiment.
FIG. 6. Bean explants on holders showing the effects of an experimental treatment.
FIG. 7. Abscissor, two views.

In later experiments, bean explants were mounted on Pyrex glass racks (explant holders) within Petri dishes (figs. 5, 6, 8). A holder consists essentially of a horizontal glass rod supported by feet and with a row of five glass pins projecting from each side. Two per cent. agar is poured around the feet of the holder to prevent displacement and to maintain a high relative humidity within the Petri dish. Dishes of explants are kept in the dark at 25° C except for the short daily period of examination.

In some cases the explants are examined through the cover of the Petri dish. In other cases the cover is raised slightly and a standard pressure applied to the free end of each explant. This pressure is applied with an abscissor (fig. 7), a device employing the principle of the spring balance. By means of the calibrated displacement of its straight spring the investigator is able to apply a known force to the explant. The instrument was developed in order to produce stresses on the abscission zone similar to those ordinarily present under field conditions, and to provide a means for apply-

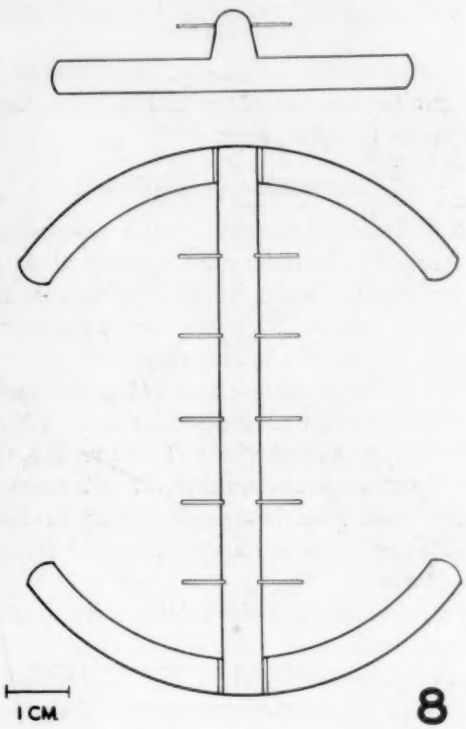

FIG. 8. Pyrex glass explant holder, two views.

ing a known force to the explants at regular intervals. Since, in the beans investigated, the leaflet weight is about one gram, the abscissor was adjusted to give a force of two grams, thus approximating the stress on the abscission zone in the field.

The technique described is a valuable tool for the investigation of leaf abscission under controlled conditions. In addition it gives promise of being adaptable to experiments with the abscission of flowers, fruits and stems.

We wish to acknowledge with gratitude the capable cooperation of Mr. H. Wilder Tomlin who constructed the explant holders and abscissor.

DIVISION OF BOTANY
UNIVERSITY OF CALIFORNIA
LOS ANGELES, CALIFORNIA

RELATION OF WATER TO THE GERMINATION OF BEAN SEEDS

ISABEL ELIZABETH STILES

(WITH FOUR FIGURES)

Received November 1, 1948

Introduction

This study is a continuation of one which appeared in a recent publication (1). For detailed account of the introduction, history of literature, etc., reference is made to that paper.

Materials and methods

A quantitative study of the course of water absorption by seeds during germination was made (1) to determine whether seeds actually differ in the total amount and rate of water intake; (2) to note the role played by the various parts of the seeds; (3) to determine whether there exist seeds with mesic (intermediate water), hydric (high water), or xeric (low water or desert) germination modifications; and (4) to determine whether there are varietal differences in water absorption by seeds. Many types of seeds have been studied, but, for the purposes of this report, the data are given only for the seeds of various beans studied. The bean seeds used are those of *Phaseolus lunatus* var. Florida Speckled Butter; *Phaseolus coccineus* var. Rubronanus; *Phaseolus vulgaris* var. Pinto or Mexican, and *Glycine Max* var. Arksoy, #2913.

The details concerning the procedure, laboratory conditions, techniques, and handling of bean seeds used were described in an earlier paper (1). Seeds of each variety were sterilized in $HgCl_2$, germinated on wet filter paper in Petri dishes at room temperature, and dissected at four 24-hour intervals of germination into their seed organs; the percentage of water was determined for like organs of each variety and expressed on absolute basis, and the percentage of water absorbed by the entire seed was determined by the same method.

Observations and results

SEED COAT.—Figure 1 shows some significant curves for Florida Speckled Butter beans. Complete hydration was obtained in 24 hours, at which time the seed coats showed a water content of 216 per cent. Throughout the rest of the period of observation these seed coats maintained approximate stability. In the air-dry seed, 7.5 per cent. of water is held by the seed coats. After 24 hours of germination this proportion increased to 12.4 per cent. With hydration of the inner parts of the seed, the percentage of total water held in the seed coat decreased.

It will be seen (fig. 2) that the seed coat of scarlet runner beans behaved similarly to those of Florida Speckled Butter beans, a state of complete hydration being reached in the first 24 hours, after which this condition was maintained with comparative stability. Scarlet runner differed from the butter beans at 24 hours in showing a lower hydration value and in displaying a gradual increase in this percentage throughout the period of observation.

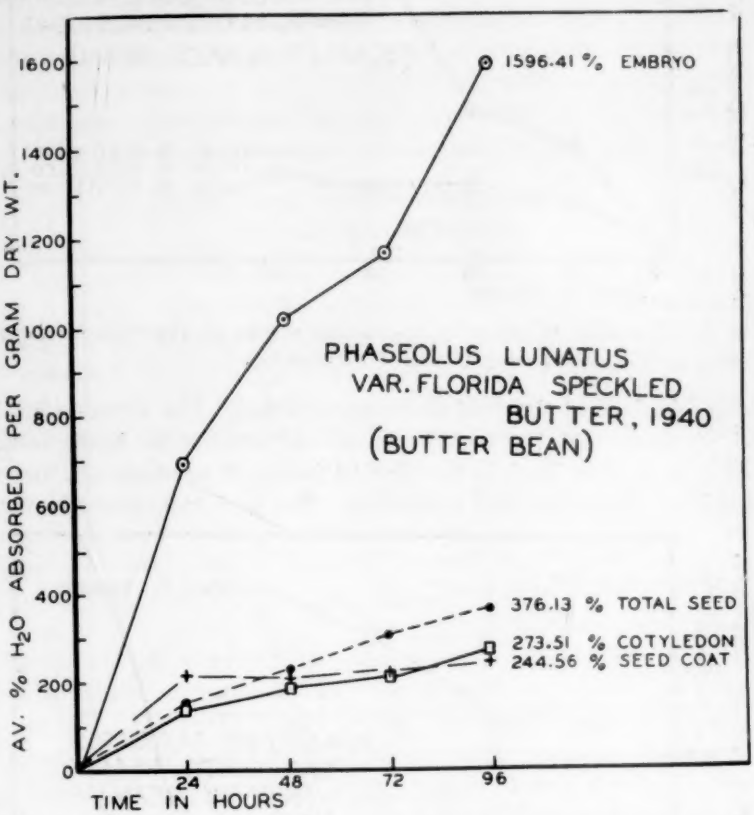

FIG. 1. Absorption of water by the various organs of *Phaseolus lunatus* var. Florida Speckled Butter beans (1940), during early phases of germination.

Figure 3 shows the curves for Pinto beans, the values of which are similar to those shown by the seed coats of Florida Speckled Butter beans. They differ from those of butter beans and particularly from those of scarlet runner beans in reaching a high maximum value in 24 hours and slowly decreasing thereafter. The proportion of total water of the seed in the seed coat decreased with hydration of the inner seed organs. Results obtained for the seed coats of soybeans (fig. 4) agree in general with those of the other varieties discussed. The behavior more closely follows that shown by scarlet runner beans, *viz.*, the maximum hydration percent-

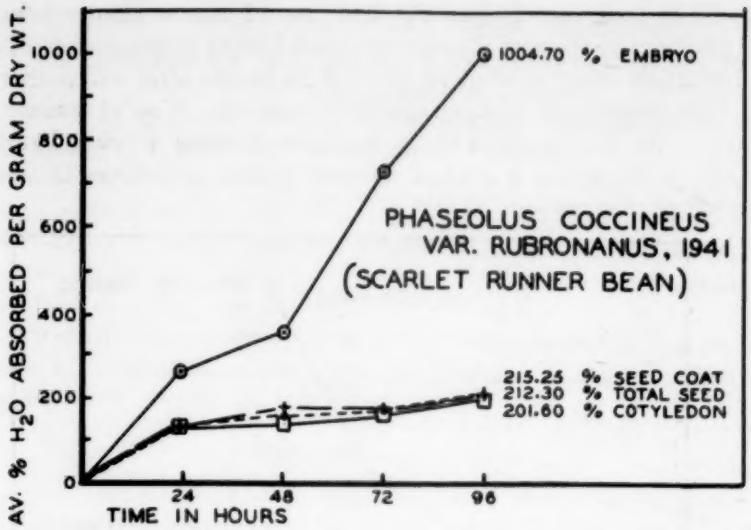

FIG. 2. Absorption of water by the various organs of *Phaseolus coccineus* var. Rubronanus (1941), during early phases of germination.

age being obtained at the final observation period. The curves (figs. 1, 2, 3, and 4) show that the seed coats of all varieties of beans observed reached a saturation level in the first 24 hours of germination, and relative stability was maintained thereafter. The seed coats play the part of

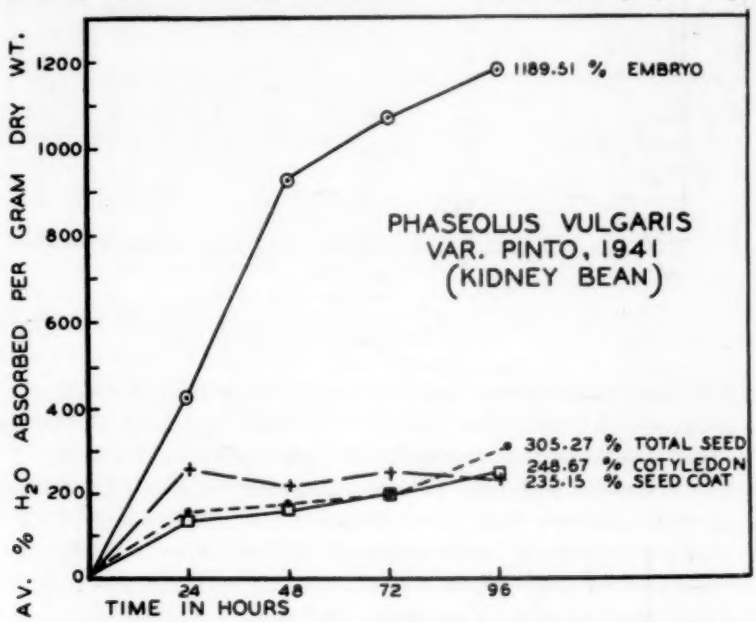

FIG. 3. Absorption of water by the various organs of *Phaseolus vulgaris* var. Pinto (1941), during early phases of germination.

transporting organs for water from the exterior environment to the interior organs of the seed.

COTYLEDONS.—Figures 1, 2, 3, and 4 present graphically the percentage relations of water in the cotyledons of the four varieties of beans. The cotyledons in beans absorbed water rapidly in the first 24 hours, and slowly but continuously thereafter. In large seeds such as those of scarlet runner and Florida Speckled Butter beans, the percentage of water contained in the hydrated food supply was greater than that observed in the smaller seeds. The highest percentage of total water in the seed during germination was contained in the cotyledons when the seeds were in air-dry condition. This indicates that these organs do not play an active part

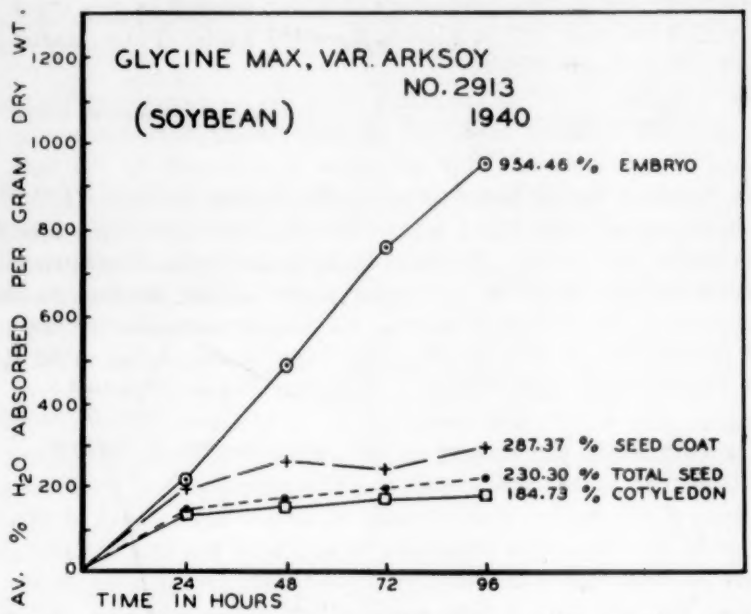

FIG. 4. Absorption of water by the various organs of Glycine Max var. Arksoy #2913 (1940), during early phases of germination.

in germinative water absorption. The cotyledons of Florida Speckled Butter, scarlet runner, and Pinto beans react alike. The cotyledons of soybeans differ from those of the other beans largely in showing a considerably lower percentage of water in the final period of observation.

The dry weight of the cotyledons of beans makes up about 88 per cent. of the dry weight of the seed. These organs consistently decreased in percentage of dry weight throughout germination, while the dry weight of the "growing parts" of the embryo increases. It is obvious that these structures are primarily food storage organs and not active absorbing structures, but that cotyledons do act as water reservoirs.

THE EMBRYO.—The organs of the embryo of seeds are the cotyledons and axis (epicotyl and hypocotyl). The cotyledons do not form any part

of the mature plant; therefore, the axis is referred to in this paper as the "growing parts" of the embryo of the four varieties of beans, Florida Speckled Butter, Rubronanus, Pinto, and Arksoy, respectively.

The percentage of water content of the "growing parts" of the embryo is higher at each period of observation than that of any other seed organ at the same period. The percentage of water in the embryo of the total water of the seed increased throughout the period of observation. The dry weight of the "growing parts" of the embryo is less than 2 per cent. of the total dry weight of the seed in the air dry condition. The dry weight of this organ increased during germination. Varietal differences appeared in the percentage of water in the "growing parts" of the embryo of the air-dry seeds. The percentage of water present in this organ was 12.20, 8.26, 15.66, and 7.27 in Florida Speckled Butter, Pinto, scarlet runner, and Arksoy, respectively.

The "growing parts" of the embryo of different varieties of bean seeds tested exhibited different capacities for water absorption during the first 24 hours of germination. This difference is evidenced by the fact that Florida Speckled Butter beans absorbed the highest per cent. (687.70%), Pinto beans second (420.41%), scarlet runner third (265.32%) and Arksoy the lowest (214.15%). The final 96 hour absorption presents varietal differences in capacity of the "growing parts" of the embryos to absorb high maximum percentages of water. Florida Speckled Butter beans absorbed the highest per cent (1596.41%), Pinto beans second (1189.51%), scarlet runner third (1004.70%), and Arksoy lowest (954.40%) at this reading.

In air-dry seeds, the embryo of all varieties of beans contained less than 3 per cent. of the seed moisture. The percentage of total water in this organ of the air-dry seed is small in proportion to that of the seed moisture in the cotyledons of air-dry seed. That the axis is the "growing part" of the embryo is evidenced by the consistent increase in the dry weight of this structure during germination, increase in size, and rapid increase in amount and percentage of water in proportion to other parts of the seed.

It is evident that all varieties of beans do not require the same amount of water for germination. Judged from both initial and final percentage of water content, the "growing parts" of the embryos of bean seeds studied show slight adaptation to germination in mesic, hydric, or xeric habitats. Arksoy beans seem to exhibit adaptation to germination in dry land (xeric) conditions. This is indicated by its low maximum water requirement for germination. This assumption is based on the fact that seed in xeric habitats would have to have a low water requirement for germination in such regions or the xerophytes would become extinct. Florida Speckled Butter beans have a comparatively high water requirement for germination and, therefore, might be assumed to be adjusted to germination in a wet land

(hydric) habitat. Seeds of Pinto beans seem to be better adjusted to germination in soil of moderate water (mesic) conditions, because of their intermediate water requirements for germination.

Summary

This is a report on a study made of the germination water relations of the seeds of *Phaseolus lunatus* var. Florida Speckled Butter, *Phaseolus coccineus* var. Rubronanus; *Phaseolus vulgaris* var. Pinto, and *Glycine Max* var. Arksoy, #2913.

This investigation indicates that various bean seeds differ in the total amount of water absorbed and in the rates of absorption; that different organs of the seed play one or more roles in the germination process; that seeds apparently possess slight degrees of adaptation to germination in mesic, hydric, and xeric conditions, and that varietal differences appear in the water absorbing capacity of seeds of the several varieties of beans tested.

The author expresses her deepest appreciation of Dr. G. W. Goldsmith (deceased) for helpful suggestions in this research work, which was conducted at the University of Texas, Austin, Texas.

HARRIS TEACHERS COLLEGE
ST. LOUIS 12, MISSOURI

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE CONTENT OF THIAMINE, RIBOFLAVIN, NICOTINIC ACID, PANTOTHENIC ACID AND CAROTENE IN STEMS AND LEAVES OF RED KIDNEY BEAN PLANTS[1]

RICHARD W. LUECKE, CHARLES L. HAMNER AND HAROLD M. SELL

Received May 16, 1949

Introduction

MITCHELL, EZELL and WILCOX (**4**) have shown that when snap bean fruits are sprayed with a water mixture of Tween 20 and p-chlorophenoxyacetic acid, the vitamin C content is increased during storage. It has also been noted by SELL, LUECKE, TAYLOR and HAMNER (**7**) that red kidney bean plants treated with 2,4-D[2] showed an accumulation of protein, amino acids, lipides and ash in the stem tissue and a reduction of carbohydrates. Reports (**6**) have been received that many farm animals prefer to eat plants treated with 2,4-D. In view of these observations, tests were undertaken to include analyses for some members of the vitamin B complex and for carotene following treatment of bean plants with 2,4-D. The results of these analyses are presented in this publication.

Materials and methods

SAMPLES OF LEAVES AND STEM TISSUE

Seeds of the red kidney bean plants were selected for uniformity of size and planted in 4-inch pots in the greenhouse. Each pot contained two plants that were treated when the first trifoliate leaf was expanding. Two replications of 100 plants each were used as a source of material for both treated and non-treated plants (controls). One drop (.05 ml.) of a 0.1 per cent. solution of 2,4-D was applied to the base of the blade of one of the primary leaves of each treated plant (**5**). The plants were harvested six days after treatment at the time the stem tissue had proliferated considerably but yet showed no signs of necrosis. The plants were dried in the dark at room temperature and finally dried in a Stokes vacuum shelf drier at 40° C for 24 hours. Analyses were made of the leaves and of stem tissue separately, the hypocotyl, first internode, and leaf petioles being grouped together as stem tissue.

ANALYTICAL METHODS

The analysis for carotene was made by the procedure approved by the A.O.A.C. (**1**). The microbiological methods employed for the determination of the B-vitamins were those of SNELL and STRONG (**9**) for riboflavin;

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1049 (n.s.).

[2] A 0.1 per cent. solution of the sodium salt of 2,4-dichlorophenoxyacetic acid acidified with orthophosphoric acid to pH 3 and abbreviated in this paper as "2,4-D."

SKEGGS and WRIGHT (8) for pantothenic acid; and KREHL, STRONG and ELVEHJEM (3) for nicotinic acid. Thiamine was determined by the fluorometric method of HENNESSY (2).

Results

The results of the chemical analyses are summarized in table I. Each entry is the average of two determinations on each of two separate samples.

The data in table I, based on micrograms of vitamin per total weight of leaves per plant, show the following trends: the leaves of the plants treated with 2,4-D contain only about one-third as much thiamine as the non-treated plants. The content of riboflavin, nicotinic acid, and carotene is also less but not to the same degree, the leaves of the treated plants containing approximately 79 per cent. as much riboflavin, 73 per cent. nicotinic acid, and 47 per cent. carotene, respectively, as the leaves of the non-treated plants. The trend for pantothenic acid is reversed when compared to the

TABLE I

B-VITAMINS AND CAROTENE CONTENT IN LEAVES AND STEMS OF RED KIDNEY BEAN PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID

VITAMIN	LEAVES		STEMS	
	TREATED	NON-TREATED	TREATED	NON-TREATED
	μg./gm./plant	μg./gm./plant	μg./gm./plant	μg./gm./plant
Thiamine	516	1570	167	89
Riboflavin	1201	1523	109	68
Nicotinic Acid	4552	6216	1294	995
Pantothenic Acid	879	516	567	223
Carotene	9432	20055	155	250

vitamins previously mentioned. The leaves of the plants treated with 2,4-D contain 70 per cent. more pantothenic acid than do the controls.

In the stem tissue, the results are somewhat different. The stems of the plants treated with 2,4-D contain in each case more vitamins than do the non-treated plants with the exception of carotene. On the basis of micrograms of vitamin per stem, the treated plants contain 88 and 60 per cent. more thiamine and riboflavin, respectively, than do the control plants. The pantothenic acid content of the stems of the treated plants is 154 per cent. more than that of the non-treated plants while the nicotinic acid is only 77 per cent. as great. On the other hand, the carotene content is only 62 per cent. that of the controls.

Summary

1. The quantity of certain vitamins in the leaves and stems of the red kidney bean plants are altered considerably upon treatment with 2,4-D.

2. The thiamine, riboflavin, nicotinic acid and carotene content is less in leaves of the red kidney bean plants treated with 2,4-D than in leaves of

the controls. Pantothenic acid is found in greater quantities in leaves of treated plants than in leaves of non-treated plants.

3. The amount of thiamine, riboflavin, nicotinic acid and pantothenic acid is much greater in stems of plants treated with 2,4-D than in stems of non-treated plants. The carotene content is approximately two-thirds as great in stems of treated plants as in stems of non-treated plants.

DEPARTMENTS OF AGRICULTURAL CHEMISTRY AND HORTICULTURE
MICHIGAN STATE COLLEGE
EAST LANSING, MICHIGAN

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NOTES

Symposia at Madison, Wisconsin, September, 1949.—An invitation is extended to all plant scientists to attend the symposia on Mineral Nutrition of Plants and on Plant Growth Substances to be held at the University of Wisconsin, September 1 to 3 and 5 to 7, respectively.

The symposia will consist of invitational papers and discussions which will review and integrate present knowledge dealing with both the scientific and practical aspects in the respective fields. The Plant Growth Substances symposium, September 5, 6, and 7 inclusive, is being conducted as part of the University of Wisconsin Centennial celebration. The symposium on Mineral Nutrition of Plants, September 1, 2, and 3, inclusive, is under joint auspices of the following societies and their representatives serving as an organizing committee: American Society of Plant Physiologists—C. H. Wadleigh; American Society of Agronomy—G. W. Volk; American Society for Horticultural Science—Damon Boynton; American Chemical Society—J. B. Hester; National Fertilizer Association —D. D. Long and M. H. McVickar; Botanical Society—R. A. Steinberg; Soil Science Society—Emil Truog. Professor Emil Truog is serving as chairman and Dr. C. H. Wadleigh as secretary. The National Fertilizer Association and other organizations are assisting with financial and social arrangements. Provision is being made for publication of papers to be presented.

Room and board will be available to participants and guests at $5.00 per person per day for the period August 31 to September 8, 1949. As a convenience to the committee on arrangements and to insure provision of the desired type of accommodations by those planning to attend, reservations should be made as early as possible. Reservations may be made for either or both symposia. For detailed programs, information about accommodations, and reservations, please write to Mr. R. P. Lee, Division of Residence Halls, Slichter Hall, University of Wisconsin, Madison 6, Wisconsin. Tentative programs of the symposia are given below.

Mineral Nutrition of Plants Symposium, September 1, 2, and 3, 1949. —Tentative program.

Section A: World food possibilities and fertility status of our soils by C. E. Kellogg and A. C. Orvedal, U. S. Department of Agriculture.

Section B: Physico-chemical and biological factors affecting nutrient availability in soils. 1. The soil as a medium for plant growth by Emil Truog, University of Wisconsin. 2. Activity of cations adsorbed on soil colloids by C. E. Marshall, University of Missouri. 3. The availability of soil anions by L. A. Dean, U. S. Department of Agriculture and Roy Overstreet, University of California. 4. Contact phenomena between plant roots and soil colloids by Hans Jenny, University of California. 5.

Effect of soil physical factors on nutrient and availability by J. B. PAGE, University of Ohio, and G. B. BODMAN, University of California. 6. The role of soil microorganisms in nutrient availability by A. G. NORMAN, Chemical Corps, U. S. Army.

Section C: Mechanism of entry and translocation of mineral nutrients in plants. 1. Transport of water and mineral nutrients by HANS BURSTRÖM, Lund University, Sweden. 2. The nature of the processes of salt accumulation by roots by D. R. HOAGLAND and T. C. BROYER, University of California. 3. Translocation of mineral nutrients by O. BIDDULPH, State College of Washington.

Section D: Field problems in plant nutrition by DAMON BOYNTON, Cornell University, and JACKSON HESTER, Campbell Soup Company.

Section E: Role of minerals in plant nutrition. 1. Essentiality of mineral elements by D. I. ARNON, University of California. 2. Mineral requirements and the ontogeny of plants by W. F. LOEHWING, University of Iowa. 3. Correlations between carbohydrate-nitrogen metabolism and mineral deficiencies by R. A. STEINBERG, U. S. Department of Agriculture.

Section F: Modifying influences of various environmental factors upon mineral metabolism. 1. Light as a modifying influence on mineral nutrition of plants by R. B. WITHROW, Smithsonian Institution. 2. Soil moisture in relation to mineral nutrition of plants by C. H. WADLEIGH and L. A. RICHARDS, U. S. Regional Salinity Laboratory, Riverside, California. 3. Environmental influences on the growth and mineral nutrition requirements of sugar cane by H. F. CLEMENTS, Castle and Cooke, Limited, Honolulu, Hawaii.

Symposium on Plant Growth Substances, September 5, 6 and 7, 1949. —Tentative Program.

General Session I—1. Isolation and chemical nature of plant growth substances by A. J. HAAGEN-SMIT, California Institute of Technology. 2. Physico-chemical properties of growth substances associated with their stimulatory and inhibitory activities by K. V. THIMANN, Harvard. 3. Biosynthesis and metabolism of growth substances by JAMES BONNER, California Institute of Technology.

General Session II—1. The formation and structure of plant cell walls by THOMAS KERR, U. S. Department of Agriculture. 2. Mechanisms of cell elongation by HANS BURSTRÖM, University of Lund, Sweden. 3. Mechanisms and chemical control of cell division in plants by A. F. BLAKESLEE, Smith College.

Evening Lectures—1. Twenty years work with plant growth substances by F. W. WENT, California Institute of Technology. 2. Some practical applications of plant growth substances by P. W. ZIMMERMAN, Boyce Thompson Institute.

General Session III—Roles of Vitamins and amino acids as growth fac-

tors for plants. 1. In bacteria by E. E. SNELL, University of Wisconsin. 2. Genetic aspects of growth responses in fungi by E. L. TATUM, Stanford. 3. Vitamin and amino acid requirements for growth of higher plants by W. J. ROBBINS, New York Botanical Garden.

Round Table Discussions—1. The role of growth substances in plant metabolism—Discussion Leaders, G. S. AVERY, JR., R. H. BURRIS, F. SMITH and K. V. THIMANN. 2. Tissue responses to growth substances—Discussion Leaders, J. M. BEAL, J. W. MITCHELL, A. R. SCHRANK, B. E. STRUCKMAYER, and P. W. ZIMMERMAN. 3. Practical applications of plant growth regulators—Discussion Leaders, K. C. BARRONS, R. S. DUNHAM, E. F. GARDNER and J. VANOVERBEEK. 4. The role of growth substances in vegetative development—Discussion Leaders, N. KENT, J. VANOVERBEEK, F. SKOOG, F. W. WENT and P. R. WHITE. 5. The role of growth substances in reproductive development—Discussion Leaders, F. G. GUSTAFSON, R. MUIR, A. E. MURNEEK, J. RAPER, R. H. ROBERTS, G. M. SMITH, and S. H. WITTWER. 6. The role of growth substances in pathological growth.—Discussion Leaders, T. C. ALLEN, R. S. DEROPP, A. C. HILDEBRANDT, I. LEWIS, G. K. K. LINK, and A. J. RIKER.

A. S. P. P. and A. A. A. S. Annual Meeting, 1949.—New York City. The American Society of Plant Physiologists is meeting this year with the American Association for the Advancement of Science and affiliated societies at New York City, December 26 to 31, inclusive. The A. S. P. P. sessions will be held December 27 to 29 inclusive. Advance registration and hotel room reservations will begin in September. Announcements and registration coupons will appear in Science. Individual notices will be sent to all A. S. P. P. members by the society secretary.

The 1950 meeting of the A. S. P. P. under auspices of the A. I. B. S. and its affiliated biological societies is tentatively scheduled for Columbus, Ohio, September 11 to 13, 1950.

New England Section.—This section of the A. S. P. P. held its annual meeting at the University of New Hampshire, Durham, May 20–21. Fifteen educational and research institutions were represented for the presentation of 22 papers. The loca' committee provided a get-acquainted hour by a midafternoon luncheon. The 70 who attended the banquet were provided a kodachrome lecture of the agriculture of Korea by DR. E. M. MEADER. The officers for the ensuing year are: Chairman, DR. R. H. WALLACE, University of Connecticut; Vice-Chairman, DR. IRENE H. STUCKEY, Rhode Island State College; Secretary-Treasurer, DR. L. H. JONES, University of Massachusetts.

Seventh International Botanical Congress, 1950.—Stockholm, Sweden. This congress will be held at Stockholm July 12 to 20, 1950 with special excursions of interest to various sections to be conducted during, be-

fore and after these dates. The program comprises fifteen sections, each devoted to a specific phase of plant science. The Organizing Committee requests those expecting to attend the Congress and to participate in excursions to file applications, not binding, in order to plan necessary arrangements. Announcements and programs may be obtained upon request from the General Secretary of the Congress, Professor EWERT ÅBERG, Uppsala 7, Sweden.

D. T. MacDougal, Honorary President, Seventh International Botanical Congress.—Stockholm, 1950.

The American Society of Plant Physiologists is pleased to announce that Dr. D. T. MACDOUGAL, a Barnes Life Member of the Society has been appointed an Honorary President of the Seventh International Botanical Congress to be held in Stockholm, Sweden, next year. Dr. MacDougal served as a member of the corporation and Director of the Laboratories of the New York Botanical Garden from 1899 to 1905. He was Director of the Department of Botanical Research and of the Laboratory for Plant Physiology of the Carnegie Institution of Washington from 1905 until his retirement in 1933. He now resides at Carmel, California.

Hunger Signs in Crops.—Second Edition. The National Fertilizer Association, 616 Investment Building, Washington 5, D. C. 370 pages. 1949. $4.50.

This revised edition presents in practical form much of the existing knowledge of symptoms that develop in growing crops which are deficient in essential plant foods. The chemical elements necessary for plant growth are discussed in the introduction of the book. Plant food deficiency symptoms in tobacco, corn and small grains, potatoes, cotton, vegetables, deciduous fruits, legumes, and citrus are each treated in separate chapters by specialists in Agronomy, Horticulture, Plant Nutrition and Crop Diseases. The new edition is more comprehensive and treats more adequately the important problems of its well known predecessor. In addition to numerous half-tone plates, the new text has 110 color plates. An appendix of special value includes a discussion of weeds, some of them are among the first plants to show hunger signs.

Introgressive Hybridization.—EDGAR ANDERSON. John Wiley and Sons, Inc., 440 Fourth Avenue, New York 16, New York. 109 pages. 1949. $3.00.

The author has succeeded in bringing together many cogent ideas, methods of experimentation and observational techniques that make the study of hybridization under natural conditions a stimulating and progressive approach to problems of evolution. Introgression is fairly clearly defined with several pertinent illustrations using species and subspecies

of Iris and Nicotiana. The ever-changing banks of the Mississippi River provide the locale for many of the careful observations and quantitative studies that support the ideas discussed. Chapter six is a lucid description of several quantitative techniques used in the study of introgression. Among those reviewed are, scatter diagrams, ideographs and hybrid indices. The genetic and cytogenetic basis for introgression are considered in some detail with emphasis on the effect of linkage upon recombination types. This small volume contains a host of current ideas that should appeal to specialists in various fields of plant science, especially taxonomy, genetics, and horticulture.

Phytotron: new experimental greenhouse opened at California Institute of Technology.—The formal dedication of the new Earhart Plant Research Laboratory on June seventh was announced by its director, Professor Frits W. Went, at the California Institute of Technology. The word phytotron was coined for the new laboratory by Dr. R. A. Millikan, former president of the California Institute of Technology, who feels that it will play the same role in plant physiology and the applied plant sciences (such as agronomy, plant breeding, forestry, horticulture, and phytopathology), as the cyclotron has already had in pure and applied physics.

The new laboratory makes it possible to grow plants under a wide variety of rigidly controllable conditions. Dr. Went states, "Heretofore many laboratories were built with one or a few temperature-controlled rooms, or with artificial light rooms, but never before were all climatic factors, such as light, temperature, humidity, gas content of the air, wind, rain, or fog, simultaneously and independently controllable."

The construction of this intricate experimental greenhouse and the type of experiments for which it will be used by Dr. Went and his associates at the California Institute of Technology, as well as by many visiting U. S. and foreign research workers, is described in detail in a booklet by Professor F. W. Went, entitled The Earhart Plant Research Laboratory, issued at the occasion of the opening of the new Plant Research Laboratory, prereprinted from Chronica Botanica, Volume 12 (Chronica Botanica Reprint, No. 2, Waltham, Massachusetts, The Chronica Botanica Co.), which will be supplied *gratis* upon request.

Erratum.—The editor's attention has been called to an error in the Notes of the April 1949 issue of PLANT PHYSIOLOGY. In the announcement of the ANNUAL REVIEW OF PLANT PHYSIOLOGY, Dr. Luck's name was erroneously given as that of the editor. The ANNUAL REVIEW OF PLANT PHYSIOLOGY is sponsored and published by the Board of Directors of Annual Reviews, Inc., Dr. Luck serving as the executive director. The annual Reviews, Inc. have also selected the editor and the editorial committee in whose hands professional affairs of the ANNUAL